Starvation Induces Expression of the Plant-Adhesin Gene, *Mad2,* of the Entomopathogenic Fungus *Metarhizium robertsii.*

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Abstract

Metarhizium robertsii is an entomopathogenic fungus that is additionally plant rhizosphere competent. Two adhesin-encoding gens, *Mad1* and *Mad2*, are involved in insect pathogenesis or plant root colonization, respectively. This study examined differential expression of the *Mad* genes for *M. robertsii* grown on a variety of insect- and plant-related substrates. *Mad1* was up regulated in response to insect cuticles and up regulation of *Mad2* resulted from root exudates, tomato stems and non-preferred carbohydrates. A time course analysis that compared water, minimal media, and nutrient rich broth revealed *Mad2* gene expression increased as nutrient availability decreased. The regulation of *Mad2* compared to known stress-related genes (*Hsp*30, *Hsp*70 and *ssgA*) under various stresses (nutrient, pH, osmotic, oxidative, temperature) revealed *Mad2* to be generally up regulated by nutrient starvation only. Examination of the *Mad2* promoter region revealed two copies of a stress-response element (STRE) known to be regulated under the general stress response pathway.

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ABC:	ATP-binding cassette
ATP:	Adenosine triphosphate
cDNA:	Complementary DNA
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
PCR:	Polymerase Chain Reaction
PDA:	Potato dextrose agar
PDR:	Pleiotropic drug resistance
PSI:	Pounds per square inch
RT:	Reverse Transcriptase
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RPM:	Revolutions per minute
v/v:	Volume/volume
w/v:	Weight/volume
YPD:	Yeast extract-peptone-dextrose broth

1. Literature Review

1.1. *Metarhizium spp.*

The control of insect pests of economically important crops has seen a shift from complete dependence on chemical pesticides, to the incorporation of biological control methods. Chemical pesticides offer a fast and effective means of control but are now limited in use as the human health and environmental ramifications outweigh the benefits (Hoppin et al. 2002; Huang et al. 2003). Biopesticides, particularly insect pathogenic microorganisms, are popularized as a new direction for pest control even though biocontrol experiments with the fungus *Metarhizium anisopliae*, for example, began over 130 years ago (Zimmermann 2007). These naturally occurring insect pathogens can be harnessed and exploited for use in crop protection, but they are currently slower acting compared to chemicals, costly to maintain and have a short shelf life (Bailey et al. 2010).

Species of *Metarhizium*, an ascomycetous fungus, are of interest since they have a biphasic lifestyle that bridges two areas of research that greatly impact the agriculture industry; as insect pathogens and rhizosphere colonizers (Zimmermann 2007; Hu and St. Leger 2002). *Metarhizium anisopliae* is currently approved for control of the black vine weevil and the strawberry root weevil in Canada and for a variety of other pests in Brazil, Colombia, Australia and the USA (Health Canada 2010; Swe et al. 2008). However, the ability of *M. anisopliae* to infect and kill the host can still take a week or longer and the survivability of the inoculum renders several applications over a season necessary (Acevedo et al. 2007; Hu and St. Leger 2002). Research has mainly focused on developing more virulent strains to decrease

the kill time but how the fungus survives in the absence of an insect host is equally, if not more important.

Species of *Metarhizium* have been isolated from soils and infected insects worldwide, with the exception of Antarctica (Zimmermann 2007). *Metarhizium spp.* are able to infect a wide-range of insects, over 200 species from seven different orders, of which the majority are soil-dwelling pest (Veen 1968). The majority of species have this broad host range (i.e. *M. robertsii, M. anisopliae*) but some are specialized at infecting certain species, such as the locust pathogen, *M. acridum* (Wang and St. Leger 2005). The recently described symbiotic association of *Metarhizium spp.* with the roots of plants appears more restricted than the association with insects. Certain *Metarhizium* species (i.e. *M. robertsii*) associate almost exclusively with grasses and wildflowers while others are predominantly found with trees and shrubs (i.e. *M. brunneum, M. guizhouense*) (Bidochka et al. 2001; Wyrebek et al. 2011). The genus *Metarhizium* was defined by the asexual reproductive structures until molecular techniques became available, as no sexual stage in nature was observed (Bidochka et al. 2001). *Metarhizium spp.* are filamentous fungi belonging to the phylum Ascomycota that grow as a network of hair-like structures (hyphae) within the soil and reproduce via mitotic spores called conidia. Conidia are produced in chains from specialized conidiogenous cells called phialides, which are terminal swellings of aerial hyphae called conidiophores (Adams and Wieser 1999). The conidia of *Metarhizium robertsii* are characteristically dark green in colour, rod-shaped, sticky and hydrophobic, and about 5-8μm in length and 2-3μm wide (Glare et al. 1996; Zimmermann 1993). Conidia rely on rainwater and insect migration for

dispersal due to the soil-borne nature of this fungus (Zimmermann 2007). The hydrophobicity of *M. robertsii* conidia allows passive adherence to the hydrophobic cuticles of both insects and plant roots (St. Leger 1993; Wang and St. Leger 2007). The ability of *M. robertsii* to moderate a pathogenic lifestyle with a variety of insects and a symbiotic association with plant roots, in addition to its global distribution, highlights why this fungus is one of the top two currently employed biological pesticides worldwide (Zimmermann 2007).

1.2. The Rhizosphere

1.2.1. Rhizospheric Competence

Rhizosphere competence is defined as the ability of a microorganism to colonize and persist within the rhizosphere, the zone of soil a few millimeters from the plant root (Schmidt 1979). The rhizosphere is high in microbial activity and subject to influence from plant root exudates (Rovira 1969; St. Leger 2008). Exudates of plants are composed of various compounds such as vitamins, organic acids, flavonoids, lipids, and predominantly carbohydrates and amino acids, which are potential substrates for the rhizosphere inhabiting microorganisms (Rovira 1969). Fungi can utilize the carbohydrate sources and may be means for the plant to obtain nitrogen and phosphorous in return (Bais et al. 2006). *Metarhizium* has been found to associate with a vast number of plant species and has never been shown to negatively impact the host plant (Zimmermann 2007). These characteristics make *Metarhizium* attractive for large-scale use as a biopesticide.

Soil-dwelling insects are the ideal target population for *Metarhizium* to control as the soil is where many insect populations overwinter, deposit eggs and mature

(Quintela and McCoy 1998). In the absence of an insect host, *Metarhizium* associates with the plant rhizosphere and would be ideally situated to infect invading insects. Maximizing the ability of this fungus to maintain large populations in the soil surrounding crop roots would minimize the chemical applications required over a given season, cutting costs and labor. Additionally, in terms of the safety of using *Metarhizium spp.* for biocontrol, the effect on non-target organisms is minimal (Zimmermann 2007).

1.2.2. Interactions with Plant Roots

Sensing the Environment

For a fungus to develop a symbiotic or pathogenic association with the roots of a plant, it must first locate the host plant. Fungi, like other microorganisms, can do so by sensing the environment and responding to cues, such as signal molecules and nutrient gradients. Mycorrhizal species of the genus *Gigaspora* are induced by plant-released compounds (unidentified "branching factors") to undergo hyphal branching, an essential trigger for the establishment of contact between hyphae and plant roots in order to develop their symbiosis (Bais et al. 2006). Soluble compounds, such as carbohydrates and flavonoids, diffuse away from the root as they are released, creating a concentration gradient. This gradient is a means for the microorganism to locate the rhizoplane (root surface) (Uren 2007).

Conidial Adhesion

Fungal attachment to the host is of fundamental importance to the successful establishment of the symbiosis as much as it is with regards to insect pathogenesis.

Insect pathogenesis by *Metarhizium spp.* is initially mediated by the attachment of the fungal conidium by reversible hydrophobic interactions via conidial surface hydrophobin proteins (i.e. SSGA, encoded by *starvation stress gene* A or *ssg*A) (St. Leger et al. 1992; St. Leger 1993; Bidochka et al. 2001). This initial hydrophobic association is subsequently reinforced with adhesin proteins (i.e. MAD1, *Metarhizium adhesin-like protein* 1) (Wang and St. Leger 2007). Hydrophobins allow passive attachment to numerous hydrophobic surfaces and this attachment is reinforced if the substrate is nutritionally acceptable (Moore 1998). Adhesins anchor the fungus to the insect cuticle in preparation for invasion (Wang and St. Leger 2007). Infection structures, an appressorium and penetration peg, are produced and in conjunction with digestive enzymes, breach the cuticle allowing the fungus to enter the insect hemolymph (St. Leger 1993). Through the action of toxins and nutrient depletion, the insect dies and the fungus emerges and conidiates on the surface of the insect cadaver (St. Leger 1993).

Rhizospheric interactions of *Metarhizium* are still in the preliminary stages of study. The mechanism of plant root adhesion is also likely to be initiated by low molecular- weight and hydrophobic, extracellular polypeptides (hydrophobins). In *Metarhizium robertsii* (ARSEF 2575) a hydrophobin gene, *ssgA,* has been characterized and is transcribed under nutrient deprivation, an activity often coinciding with conidiation and formation of infection structures (St. Leger et al. 1992).

MAD2 Adhesin Protein

MAD2, *Metarhizium* adhesin-like protein 2, is involved in conidial attachment to plant material (Wang and St. Leger 2007). MAD2 is structurally similar to *Candida albicans* cell wall agglutinin-like sequence (ALS) proteins, enabling stable H-bond-dependent connections with peptides of the host surface (Wang and St. Leger 2007). The N-terminal domain of MAD2, a hydrophobic signal peptide, and the C-terminal region, a predicted glycosylposphatidylinositol (GPI) anchor, flank three tandem-repeat sequences that are believed to produce a rigid elongation structure that holds the N-terminal end at the surface of the conidium (Wang and St. Leger 2007). These studies also showed that MAD2 is absent on hyphal bodies and is detectable on conidia once they swell in preparation for germination. Deletion mutants for *Mad2* resulted in conidia that were unable to adhere to the epidermis of onion, illustrating that MAD2 is essential for *Metarhizium* to irreversibly adhere to plant material (Wang and St. Leger 2007). *Metarhizium robertsii* has been shown to not produce appressoria on plant material but it does invade the intercellular space of the superficial cell layers supporting the idea that this interaction is not pathogenic in nature (Fang and St. Leger 2010; Sasan and Bidochka, personal communication). This endophytic type of growth (Hartig net) is also found in ectomycorrhizal fungi such as *Pisolithus tinctorius*, whose hyphae penetrate the root cuticle of *Eucalyptus pilularis*, separate living cortical cells at the middle lamella and extend intercellularly receiving carbon nutrition in exchange for nitrogenous and phosphorous compounds (Smith and Read 2008).

The *Mad* genes were initially identified by differential gene expression profiles when *Metarhizium robertsii* was grown in media containing insect cuticle, hemolymph or bean root exudate (Wang et al. 2005; Wang and St. Leger 2007). *Mad2* was up regulated when *M. robertsii* was grown with bean root exudate while *Mad1* was up regulated with insect cuticles (Wang et al. 2005). The specific components of root exudate that up regulate the *Mad2* gene are undetermined.

1.3. Plant Root Exudates

1.3.1. Naturally Occurring

Numerous factors affect the composition of plant root exudates; plant species, plant age, temperature, light, nutrient availability, microorganisms, moisture and root damage (Rovira 1969). Any of these factors will alter the composition of the root exudate that will in turn affect the surrounding rhizosphere. The highest amount of exudate is released from seedlings and decreases as the plant ages (Rovira 1969; Vancura and Stanek 1975). The release of different compounds or the same compound but in different concentrations will affect the composition of the rhizosphere and thus the microbial activity within it. It is estimated that 5% to 21% of all photosynthetic carbon is transferred to the rhizosphere amounting to a significant carbon loss to the plant (Walker et al. 2003). Up to 40% of root exudate is carbohydrates, of which glucose, fructose, xylose, arabinose, sucrose and galactose are the most prevalent (Buxton 1967; Jalali and Suryanarayana 1971).

The quantity of carbohydrates available to, and utilizable by, *Metarhizium* could affect its survival within the rhizosphere. Non-preferred sugars, such as lactose,

raffinose, fructose, arabinose and sorbose could cause nutritive stress for the fungus (Rangel et al. 2006; St. Leger et al. 1994). Slower utilization of less preferred substrates and the energetic costs required to produce enzymes to break them down could be consequential in the colonization of the plant rhizosphere by *Metarhizium.*

Glucose is an easily metabolized sugar for fungi and is a major byproduct of photosynthesis (Griffin 1981). The plant utilizes glucose for energy through respiration, in the production of cellulose for its cellular material and in production of starch as energy storage material (Griffin 1981). Analysis of the exudate of wheat during the presence and absence of infection by *Helminthosporium sativum*, showed the quantitative and qualitative differences that arise in the carbohydrate spectrum (Jalali and Suryanarayana 1971). This study showed that glucose, fructose, xylose, arabinose and galactose are quantitatively the most abundant carbohydrates released into the root exudate when the plant is healthy. In plants infected with *H. sativum* ribose, maltose, raffinose and sucrose were found in greater abundance when compared to exudates of healthy plants. Quantitatively, release of carbohydrates was 1.5 times higher from healthy plants than infected plants (Jalali and Suryanarayana 1971).

Many carbohydrates, amino acids and organic acids are ubiquitous in root exudates and thus unlikely to act as specific signaling molecules for rhizospheric microorganism. Less abundant compounds are more likely to act as specific signaling molecules (Rovira 1969; Czarnota et al. 2003). However, Hildebrant (2006) demonstrated that raffinose, a common root exudate carbohydrate, was effective at stimulating hyphal growth in the arbuscular mycorrhizal fungus, *Glomus intraradices.*

Rhizospheric competence of *Metarhizium* relies on its ability to transport and utilize a variety of plant-derived carbohydrates. Fang and St. Leger (2010) identified a gene, denoted *Mrt* (*Metarhizium* raffinose transporter) that encoded a transporter that had a high affinity for heterologous oligosaccharides and disaccharides, such as raffinose and lactose. This transporter appeared to be exclusively responsible for transporting heterologous sugars. Their results showed that monosaccharides (i.e. glucose) and glucooligosaccharides (i.e. trehalose and maltose) did not antagonize the uptake of raffinose. The growth of *M. robertsii Mrt* deletion mutants on media containing raffinose (or other heterooligosaccharides) was significantly impaired compared to the wild type (Fang and St. Leger 2010). Germination of Δ*Mrt* mutant conidia less than 3 mm from grass roots was significantly lower than that of the wild type and mutant germlings failed to develop any lateral hyphal branches. Hyphal branching is an indication of successful rhizosphere colonization (Bais et al. 2006; Harrison 2005).

Fang and St. Leger (2010) constructed an *Mrt-GFP* (green fluorescent protein) transformant where the expression of GFP was under the control of the *Mrt* promoter. Their work showed that the distribution of utilizable heterologous oligosaccharides was limited to within 3 mm from the rhizosphere and that rhizosphere competency of the Δ*Mrt* mutant was reduced. After 3 months from the time of inoculation, the number of CFUs of the wild type was 11-fold greater than that of the Δ*Mrt* mutant in the rhizosphere (Fang and St. Leger 2010). Therefore, the ability to transport various carbohydrates plays a significant role in the survivability of symbiotic fungi within the rhizosphere. Fungal transporters required for rhizospheric competency have not been

fully elucidated in rhizospheric fungi. An exception is the monosaccharide transporter of *Geosiphon pyriformis*, which is implicated in plant symbiosis (Schüssler et al. 2006; Fang and St. Leger 2010).

Some plant carbohydrates may be antagonistic to fungi. For example, sorbose is toxic to many fungi (Brockman and De Serres 1963; Rangel et al. 2006) and could act as a potential antagonist against pathogenic fungi. When garlic root exudate was infused into PDA plates and inoculated with the oomycete *Phytophthora capsici*, significant inhibition of mycelial growth was observed, proportional to the concentration of exudate used (Khan and Zhihui 2010). Exudate from mutant tomato plants impaired in their ability to initiate association with *Gigaspora gigantea* and *Glomus intraradices*, inhibited hyphal proliferation in both fungal species unlike exudate from wild-type tomato plants (Gadkar et al. 2003). The varying conditions of the rhizosphere would thus be able to exclude certain species, such as pathogens, and promote beneficial colonizers. The findings that certain species of *Metarhizium* (*robertsii* and *brunneum*) are found typically with certain plant types (grasses/wild flowers and shrubs/trees, respectively) in Ontario (Bidochka et al. 2001b; Wyrebek et al. 2011) supports the idea that plant exudates and thus plant species could influence fungal rhizosphere fitness.

An aspect of plant-fungal symbioses is the ability of the plant to produce chitinases, peroxidases, reactive oxygen species (ROS) and other pathogen-related proteins, that limit fungal growth to prevent the association from becoming parasitic in nature (Sirrenberg et al. 1995; Knogge 1996). Evolutionarily the prolonged interaction of a fungal symbiosis is preferential to the plant as it gains access to

organic compounds and minerals provided by the fungus. The fungus, rather than executing short-lived parasitism, receives a longer lasting supply of plant-derived carbon in an intimate association with the plant root (Martin and Tagu 1999). Although this appears to be an optimal arrangement, this symbiotic association always has the potential to become parasitic and could be rejected by the plant (Baron and Zambryski 1995).

Non-self factors such as chitin- and glucan-fragments on the fungal surface, fungal secreted proteins or plant self-determinants released due to invasion and activity of hydrolytic enzymes signal to the plant a potential pathogen (Knogge 1996). The subsequent response of the plant subjects the fungus to a variety of antifungal elements to which the fungus must respond in order to succeed in establishing an association. These components are released in response to the perception of a threat, but what about exudates that are collected aseptically?

1.3.2. Aseptically Collected

Collecting exudates from aseptically grown plants is said to give an understanding of the true nature of root exudates (Rovira 1965). The complication, as already mentioned, is that the nature of the exudate changes according to the microflora of the rhizosphere and thus *in vitro* experimental results are sometimes limited in their applicability. Exudates are collected by various means such as localized sampling, solution- or soil-culture systems or (frequently) by means of a trap solution (Neumann and Romheld 2007). Roots of a plant or seedlings are submerged in sterile water or nutrient trap solution into which the exudate is released. The

solution can be filtered, concentrated by vacuum evaporation or freeze-drying, and used in experiments at a standard concentration. Concentrating the exudate is often necessary, depending on plant species: seven times as much exudate from oats compared to pea was required to yield similar detectable quantities of carbohydrate (Rovira 1965).

The difficulty in determining an ecologically relevant concentration of root exudate is that a spatial gradient exists along the longitudinal axis of the root; the consensus is that the highest concentration is at the root tip (Rovira 1969; Pearson and Parkinson 1961; Wenzel et al. 2001). Most recent data suggests that reconstituting dried root exudate to a concentration between 0.01-0.1 mg/mL is representative of rhizospheric conditions (Wenzel et al. 2001; Fang and St. Leger 2010).

These aseptically collected exudates, although void of pathogen-induced defense compounds, still contain compounds that can be toxic to various organisms. Pava-Ripoll et al. (2010) found a putative ATP-binding cassette (ABC) transporter gene, through EST (expressed sequence tag) analysis, that was highly up regulated when *M. robertsii* was grown for 12 hours in aseptically obtained bean-seedling exudate. ABC transporters are evolutionarily conserved membrane proteins found in all orders of life and are especially known for the efflux of toxic compounds from a cell (Sturm et al. 2009).

1.4. The Stress Response

The energy expenditure required responding to both stimulatory and inhibitory components of root exudate places a higher demand on the fungus for energy-

containing metabolites, creating nutritive stress. There is a phenomenon that occurs in fungi and bacteria where exposure to one type of stress causes a subsequent increase in resistance to other unrelated stresses and is termed stress-induced cross-protection (Völker et al. 1992; Schüller et al. 1994; Rangel 2010). Rangel et al. (2006) was able to show that growth of *M. robertsii* on non-preferred carbohydrate sources yielded conidia with greater UV-B resistance. This phenomenon has also been shown in *S. cerevisiae* and *C. albicans* (Mitchel and Morrison 1982; Quinn 2008; Rangel et al. 2008), and is explained by the overlap that occurs between the various stress-response pathways.

1.4.1. Transcriptional Level Control

The best-characterized response to stress in eukaryotes as well as prokaryotes is the production of stress response proteins and their regulation via transcription factors (TFs). TFs are DNA binding proteins that bind alone or in a complex with other proteins to specific nucleotide sequences adjacent to genes (Karin 1990; Latchman 1997). The binding of TFs either promotes or blocks the recruitment of the RNA polymerase enzyme, thus regulating gene transcription. While other proteins such as co-activators, chromatin remodelers, histone acetylases, deacetylases, kinases and methlases play important roles in gene regulation, they lack a DNA-binding domain and are not considered transcription factors (Brivanlou and Darnell 2002). Gene regulation is more complex than just the action of TFs, involving mRNA splicing and stability, translation and post-translation events but will not be covered here (reviewed by Day and Tuite 1998).

The entire genome of the model yeast *Saccharomyces cerevisiae* has been sequenced, providing an invaluable tool to elucidate the mechanisms involved in the transcriptional events leading up to the production of stress response proteins. As the response to stress was highly conserved amongst all divisions of life, discovering the mechanisms in a simple organism such as *S. cerevisiae*, allows comparison to other fungal species (Lengeler et al. 2000; Bahn et al. 2007). The transcription factor encoding genes discussed herein are of *S. cerevisiae* unless noted otherwise.

Toxic Substances

The activation of transcription of genes encoding ABC-transporters in response to the presence of toxic compounds is regulated by the binding of transcription factors to conserved sequences within the promoter region. One such binding site is the *cis*-acting PDRE (Pleiotropic drug resistance element) with consensus sequence 5'-TCCG/aC/tGG/cA/g-3' (Wolfger et al. 2001). The largest and best characterized of the ABC-subfamily is the pleiotropic drug resistance (PDR) subfamily of *S. cerevisiae*. These large transmembrane spanning proteins are able to pump out hundreds of structurally and functionally unrelated compounds in an adenosine triphosphate (ATP)-dependent manner (Wolfger et al. 2001).

The genes for these proteins are under the regulation of both PDR factors (i.e. Pdr1p/Pdr3p) as well as other stress response factors such as Yap1p and Msn2/Msn4 (Wolfger et al. 2001). Yap1p belongs to the yeast activating protein (Yap) family of TFs and mediates activation by binding to the yeast Ap-1 response element (YRE) sequence 5'-TTAG-3', or its inverse 5'-CTAA-3', and to the general control nondepressible (GCN4) element 5'-TGACTCA-3' (Fernandes et al. 1997). GCN4

positively regulates genes expressed primarily during amino acid starvation but also those involved in purine and organelle biosynthesis, autophagy, glycogen homeostasis and multiple stress responses (Hinnebusch 1992; Natarajan et al. 2001).

Multiple Stress Response: The STRE

Msn2 and Msn4 (Msn2/4) are partially redundant, *trans*-acting, zinc-finger transcription factors involved in activating genes with the *cis*-regulatory element STRE (stress response element) (Estruch and Carlson 1993; Martinez-Pastor et al. 1996; Gorner et al. 1998). The STRE has the sequence, 5'-AGGGG-3', is also functional in the inverse, CCCCT, and can occur numerous times within the promoter of a single gene (Marchler et al. 1993). Msn2/4 respond to various stressors including glucose starvation, during a diauxic shift, heat shock, and oxidative- (H_2O_2), osmotic- and acid stress (Martinez-Pastor et al. 1996).

The STRE appears to be a convergent point for at least three pathways (See Figure 1): the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway, the target of rapamycin (TOR) pathway, and the mitogen-activated protein kinase/high osmolarity glycerol (MAPK HOG) pathway, lending a plausible explanation for the cross-protection phenomenon (Estruch 2000). The response of the transcription factors Msn2/Msn4 to various stresses through each of these pathways is discussed below.

1.4.2. Pathway Cross-talk

Cyclic AMP and Catabolite Repression

The cAMP-PKA pathway negatively regulates a variety of stress-induced genes. This pathway is involved in nutrient sensing, regulation of cell growth and proliferation and in the stress response (Estruch 2000). cAMP is produced from the activation of adenylate cyclase and activates the cAMP-dependent protein kinase A (PKA). Binding of

cAMP to the regulatory subunit of PKA causes release of several catalytic subunits that phosphorylate many target proteins (Estruch 2000). The activation of adenylate cyclase can either occur through Ras proteins or by the G protein-coupled receptor system.

Ras proteins (small G proteins) are active when bound to GTP, stimulating adenylate cyclase and a rise in cAMP, and inactive when GTP is hydrolyzed to GDP (Tamaki 2007). The Ras proteins have been described as having a prominent role in response to stress conditions that result in the accumulation of misfolded proteins, due to the upstream regulation of the 70kDa heat shock protein (Hsp70), Ssa1p, on the guanine nucleotide exchanger protein, Cdc25p (Estruch 2000; Geymonat et al. 1998). In unstressed conditions, Ssa1p was shown to co-immunoprecipitate with Cdc25p (Geymonat et al. 1998). This physical interaction positively regulates Cdc25p that in turn activates the Ras proteins (Ras1p/Ras2p) to activate the cascade resulting in cAMP production. A rise in misfolded proteins during stress events would recruit the activity of Ssa1p causing a disassociation with Cdc25p, thus derepression of genes under the regulation of the Ras-cAMP pathway (Estruch 2000).

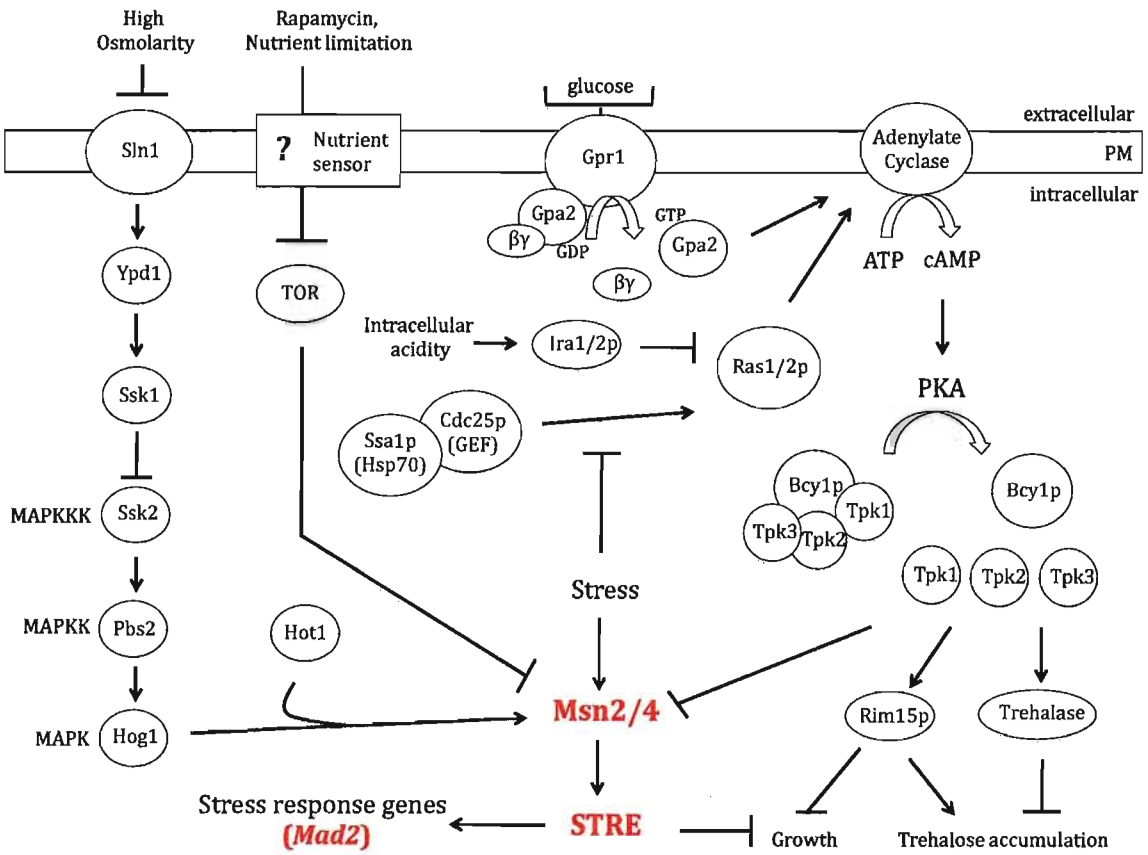

Figure 1. Convergence of several pathways characterized in *Saccharomyces cerevisiae* on the stress-response element (STRE) through the action of the transcription factors Msn2 and Msn4 (Msn2/4). *Mad2* is the theoretical placement of the *Mad2* gene of *M. robertsii* (not characterized in *S. cerevisiae*) assuming similar regulatory pathways as they are highly conserved amongst eukaryotes. Arrows represent activation and blunt-end lines represent inhibition. PM: plasma membrane (Adapted from, Thevelein and de Winde 1999; Estruch 2000; Versele et al. 2001; O'Rourke et al. 2002; Bahn et al. 2007).

In addition to responding to stress, the Ras-cAMP pathway appears to respond to the presence of glucose. When *S. cerevisiae* is supplied glucose, there is a measurable spike in the level of cellular cAMP absent in a triple-mutant strain (*Δras1, Δras2, Δpde2*) and in a mutant lacking the guanine nucleotide exchanger protein (Mazon et al. 1982; Mbonyi et al. 1988; van Aelst et al. 1991). This mode of action is believed to occur from derepression of the Ira (Inhibitor of Ras) proteins. Metabolism of glucose produces ATP and the intracellular compartment pH remains homeostatic. Glucose limitation and thus ATP depletion acidifies the intracellular compartment, inducing the Ira proteins (Colombo et al. 1998). The Ira proteins negatively regulate Ras proteins and thus inhibit cAMP production (Colombo et al. 1998; François and Parrou 2001).

Glucose-induced production of cAMP is also accomplished by a G-protein coupled receptor system (Estruch 2000; Bahn et al. 2007). Extracellular glucose is sensed by the extracellular receptor (Gpr1) that stimulates GDP-GTP exchange on the cytoplasmic-residing protein, Gpa2. Gpa2 is an activator of adenylate cyclase and thus the cAMP-PKA pathway (Bahn et al. 2007). The transient increase in cellular levels of cAMP repress the expression of genes that would otherwise halt the growth of cells, cause entry into the stationary phase and induce utilization of glycogen- and trehalose stores due to glucose limitation (François and Parrou 2001).

Target Of Rapamycin

The central cAMP-PKA pathway is one mechanism by which the STRE-binding TFs, Msn2/4, are inhibited; PKA-directed phosphorylation prevents their nuclear localization (Görner et al. 1998). Hyperphosphorylation of Msn2/4 has been

shown to occur in response to heat shock and at the diauxic shift illustrating antagonism between PKA and stress-activated kinases (Garreau et al. 2000). The ability of Msn2/4 to respond to various stresses may be due to different hyperphosphorylation patterns by various stress-activated kinases (Garreau et al. 2000). Msn2/4 are retained in the cytoplasm by being bound by the 14-3-3 regulatory protein, Bmh2p, and are released in response to nutrient (i.e. glucose, nitrogen) limitation (Roberts et al. 1997). Once Msn2/4 have exerted their transcriptional activity they must be exported from the nucleus by the export receptor Msn5 (Estruch 2000). Cytoplasmic-retention of transcription factors is a means for a cell to prevent transcription of genes at the incorrect time. A pathway that utilizes this method for the control of starvation-responsive transcription factors is the TOR pathway (Beck and Hall 1999; Schmelzle et al. 2004).

In the absence of rapamycin and nitrogen limitation, TOR promotes the formation of a complex between the phosphatase, SIT4, and its inhibitor, TAP42 (Schmelzle et al. 2004). Without this complex formation, SIT4 dephosphorylates the transcription factor, GLN3 releasing it from its tether (URE2) and GLN3 translocates to the nucleus to induce transcription of nitrogen catabolite repressed genes (Schmelzle et al. 2004). Treatment of *S. cerevisiae* with rapamycin induces translocation of Msn2/4 to the nucleus but in a SIT4-independent manner and is retarded in cells with an activated Ras-cAMP pathway, revealing the existence of a novel intersection of the TOR and Ras-cAMP pathways yet to be discerned (Bertram et al. 1998; Schmelzle et al. 2004). The transcriptional element PDS (post diauxic shift element), with sequence: T/AAGGGA, is highly similar to the STRE. The PDS

element is also induced by nutrient limitation but unlike the STRE, it is not induced by other stressors (i.e. osmotic stress, heat shock) and is Msn2/4-independent (Boorstein and Craig 1990; Pedruzzi et al. 2000).

HOG-MAPK

Osmotic stress can cause decreased growth and viability, severe loss of the plasma membrane gradient, and water, and can compromise the cell's structural integrity by shrinkage (Mager and Varela 1993; Toone and Jones 1998). Cells respond to osmostress by increasing intracellular glycerol concentrations and thus their intracellular osmolarity (Blomberg and Adler 1989). The HOG-MAPK pathway is essential in maintaining water content and is shown to also require Msn2/4.

The work done by Posas et al. (1996) found that osmolarity is sensed by the transmembrane protein Sln1 and under stable osmotic conditions remains constitutively active. When active, the intracellular histidine kinase domain phosphorylates the protein Ypd1 that then transfers its phosphate to the regulator protein Ssk1. Ssk1 is unable to interact with the MAPKKK thus inhibiting the remaining kinase cascade (Posas and Saito 1998). High external osmolarity inhibits the activity of Sln1 leaving Ssk1 dephosphorylated and able to interact with MAPKKK. The MAPKKK undergoes autophosphorylation and then phosphorylates the MAPKK, which subsequently phosphorylates Hog1 (the final kinase; MAPK) (Posas and Saito 1998). Msn2/4 and Hog1 are translocated separately to the nucleus after osmotic stress and Msn2/4 secures Hog1 within the nucleus allowing Hog1 to recruit another TF, Hot1, inducing transcription (Westfall et al. 2004).

Heat Shock Response

STREs are common in the promoter of genes induced by heat stress in addition to the heat shock element (HSE) sequence, 5'-nGAAn-3'; characterized by at least three contiguous, inverted repeats (Amin et al. 1988; Fernandes et al. 1994). The HSE is the binding site for the heat-shock factor (HSF). The amino-terminal activation domain of the HSF is responsible for heat shock response while its carboxy-terminal activation domain regulates transient responses to other stresses and helps to maintain sustained responses (Sorger 1990). The HSF is maintained in an inactive monomeric form by Hsp70 during non-stress conditions in both the cytosol and nucleus, much like Cdc25p, and becomes a trimer upon temperature elevation as well as other stresses (Knauf et al. 1996; Kline and Morimoto 1997).

The HSE was primarily associated with a family of proteins discovered to actively respond to heat shock treatment, conserved in both bacteria and eukaryotes - the heat shock proteins (HSPs) (Lindquist 1986; Lindquist and Craig 1988). The HSPs function as molecular chaperones that assist in protein folding, assembly, translocation and degradation even under non-stressed conditions (Parsell and Lindquist 1993). As newly synthesized polypeptides emerge from ribosomes, these chaperones (i.e. Hsp70) reversibly bind via hydrophobic residues to prevent their aggregation. Once the entire polypeptide is synthesized and begins to fold, an exchange of ADP for ATP inactivates the chaperone and causes the release of the polypeptide in a controlled manner (Hinault and Goloubinoff 2007).

Other members, such as Hsp30, are hydrophobic plasma membrane associated and are responsible for the negative regulation of H+-ATPase activity induced by heat

shock, organic acids, ethanol, glucose limitation and entry into stationary phase (Panaretou and Piper 1992; Piper et al. 1997). HSPs work in concert to unfold and refold protein aggregates in an ATP dependent manner. The Hsp104 chaperone forms an active hexameric ring structure that can pry apart protein aggregates, making the hydrophobic surfaces once again accessible to other members like Hsp40 and Hsp70, to facilitate refolding (Glover and Lindquist 1998; Lum et al. 2004). In the event of heat shock, there is a transient increase in the transcription of these proteins, which act to prevent and repair protein denaturation and aggregation (Martin et al. 1992; Wu 1995). The heat shock response can now be viewed as a general protein-damage response that, much like the STRE driven responses, unites a variety of unrelated stressors to overall enhance the survivability of an organism. The regulation of genes responsive to different types of stresses, even though they contain these generalized elements (STRE, HSE), must then be reliant on the context of the entire promoter sequence, encompassing numerous transcriptional elements and regulatory sequences (Estruch 2000).

The objective of this study was to examine the regulation of the gene encoding the plant-adhesin-like protein 2 (*Mad2*) of *Metarhizium robertsii*, previously shown to be up regulated in bean root exudate. The expression of *Mad2* was to some degree compared to *Mad*1, the insect-adhesin encoding gene, as they were previously found to be differentially expressed. This study aimed at identifying the component of root exudate that induces *Mad2* expression by examining the transcriptional effect of various carbohydrates (primary constituent of root exudate) and comparing the effect of exudates of different plant species on the expression of *Mad2*. An analysis of

Mad2 gene expression over the course of 72 hours for *M. robertsii* grown in water, minimal media, and nutrient broth was performed, revealing the impact of stress on *Mad2* gene expression. Transformation of *M. robertsii* to express a gene construct, where expression of a green fluorescent protein was under the control of the *Mad2* promoter, was used to confirm the results of the molecular studies. Furthermore, *Mad2* gene expression was compared to several stress-related genes in order to investigate the nature of the stress (i.e. nutrient, osmotic, oxidative, heat, pH) involved in *Mad2* gene expression.

2. Materials and Methods

2.1. Fungal Strain and Inoculum Preparation

The culture of *Metarhizium robertsii* strain ARSEF 2575 originated from the United States Department of Agriculture – Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (USDA-ARSEF; Ithaca, New York). This strain of fungus was known as *Metarhizium anisopliae* var. *anisopliae* until recent taxonomic reclassification (Bischoff et al. 2009).

M. robertsii was grown on PDA (Difco; Sparks, Maryland) plates at 27°C in the dark for 10 days to allow adequate conidial production. Conidia were dislodged from the agar in 4 mL of 0.01% Triton-X and adjusted to a concentration of 1.8×10^8 conidia/mL. Of the conidial suspension, 2 mL was inoculated into 200 mL of YPD broth (Difco) in 500 mL Erlenmeyer flasks and they were shaken at 120 rpm for 4 days at 27°C. The fungal mat was washed 3 times with sterile distilled water, collected by vacuum filtration and divided into approximately 1.0 g wet weight fractions for immediate inoculation of individual experimental flasks. All experiments had inoculum prepared as described unless noted otherwise and were performed independently three times.

2.2. Media and Growth Conditions

Cuticle and Exudate Media. To 20 mL of distilled water in 50 mL Erlenmeyer flasks, one of the following was added: 1% w/v insect cuticle derived from locust (*Locusta migratoria*), the giant cockroach (*Blaberus giganteus*), or the

tobacco hornworm (*Manduca sexta*) purified by method of Anderson and Roepstorff (1978); one of three concentrations (0.1%, 1%, 10% v/v) of bean root exudate (described below); 1% w/v starch, and 1% w/v cellulose. Flasks were sterilized by autoclaving (20 minutes, 121°C, 15 PSI), inoculated with a 1.0 g wet weight fungal fraction and incubated for 12 hours at room temperature on a shaker at 120 rpm.

Carbohydrates Media. To 20 mL of distilled water in 50 mL Erlenmeyer flasks, 1% w/v of one of the following carbohydrates was added: glucose, trehalose, N-acetylglucosamine, raffinose, lactose, maltose, arabinose, sorbose, or fructose. A second series of 50 mL Erlenmeyer flasks containing 10 mL distilled water with 2% w/v of the same carbohydrates was autoclaved, then 10 mL of a separately autoclaved 0.2% solution of peptone was added to each; the final concentration being 1% carbohydrate with 0.1% peptone. Flasks were inoculated with a 1.0 g wet weight fungal fraction and incubated for 12 hours at room temperature on a shaker at 120 rpm

Exudate Collection. To obtain healthy root exudates, bean, asparagus, carrot, and basil seeds were surface sterilized by being submerged in 4% sodium hypochlorite with mild agitation for 2 hours followed by three washes in sterile distilled water. Sterile seeds were germinated on YPD agar plates and, once >95% of seeds had germinated, placed in a 15 cm diameter Petri dish and covered with 50 mL sterile distilled water. The Petri dishes were sealed with Parafilm® wax and placed on a rotary shaker at 30 rpm for 4 days at room temperature. Since the germination times for each type of seed varied, four days after germination was chosen as the standard to collect the exudate. Bean root exudate was collected by vacuum-filtration through a 0.22micron filter paper and tested for sterility by swabbing a sample of each exudate

on a YPD agar plate incubated at 27°C for 7 days. Bean root exudate was used at concentrations of 0.1%, 1% and 10% (v/v). The exudates of asparagus, carrot and basil were also collected by vacuum-filtration and tested for sterility. Exudates of asparagus, carrot and basil were frozen and lyophilized, and reconstituted with sterile-distilled water to a concentration of 0.1 mg/mL.

Damaged root exudates were collected from asparagus, carrot and basil. The same seedlings from which the healthy exudates were collected had their roots pounded with a pestle to simulate damage. These seedlings were covered with 50 mL of sterile distilled water, left to incubate for 20 minutes at room temperature, and the liquid was collected by vacuum-filtration and tested for sterility. Sterile, lyophilized exudates were reconstituted with sterile-distilled water to a concentration of 0.1 mg/mL.

Time-Course Series Media. The starvation time-course media consisted of a series of sterile-distilled water, minimal media M-100 (10 g dextrose, 3 g KNO_3, 62.5 mL of M-100 salt solution per 1 L; M-100 salt solution: 16 g KH_2PO_4, 4 g Na_2SO_4, 8 g KCl, 0.9857 g $MgSO_4$, 1 g CaCl2, and 8 mL of M-100 Trace Element Solution per 1 L; Trace Element Solution: 60 mg H3BO3, 140 mg $MnCl_2 \cdot 4H_2O$, 400 mg $ZnCl_2$, 40 mg $Na_2MoO_2 \cdot 2H_2O$, 60 mg $FeCl_2$, 400 mg $CuSO_4 \cdot 5H_2O$ per 1.0 L) or YPD broth flasks. Each was prepared at a volume of 20 mL and inoculated with a 1.0 g wet weight fungal fraction. A sample flask from each media type was collected at the following time intervals: 15 minutes, 2, 4, 8, 12, 24, 48, and 72 hours. For the time interval of 15 minutes, the inoculum was added and the flask was swirled for 15 minutes in the respective solution and then filter collected and tested. The sample for YPD was taken directly from the four-day culture and tested.

Stress Test Media. The effect of several stressors on the expression of *Mad2*, *Hsp*30, *Hsp*70 and *ssgA* were evaluated in both 20 mL YPD and 20 mL distilled water that were adjusted to the following conditions: incubation at 15°C, 22°C, 37°C, pH 4 (adjusted with 1M HCl), pH 10 (adjusted with 1M KOH), 10 mM and 100 mM H_2O_2 (added after fungal inoculation), 0.7M and 1.5M potassium chloride (KCl), 0.5M and 2M sorbitol, and 1% *Manduca sexta* cuticle. All media was filter sterilized (with the exception of *Manduca sexta* cuticle which was autoclaved) with 0.22micron filter paper and prepared the day of the experiment. All samples were incubated on a shaker for 2 hr at 180 rpm at room temperature unless noted otherwise.

2.3. Extraction of RNA

Cultures were filter-collected with diethyl-pyrocarbonate (DEPC)-treated materials to inhibit RNases, placed onto labeled pieces of autoclaved aluminum foil and immediately stored at -80°C. Each sample was crushed under liquid nitrogen in a mortar and pestle that had been treated for RNase contamination. Total RNA was extracted from cultures grown in media containing insect cuticles, bean root exudate, chitin, tomato stem, starch, or cellulose using TRI reagent (Sigma-Aldrich Canada Ltd.) as per the manufacturer's instructions. For all other samples, the RNeasy Plant Mini Kit (Qiagen; Mississauga, Ontario) was used as it was faster for the volume of samples and did not involve the use of the toxic chemicals phenol and chloroform.

RNA from each sample was checked for stability by agarose gel electrophoresis. 5uL of RNA was run on a 1.5% agarose gel in 0.5X TBE buffer (Tris-boric acid-EDTA) using ethidium bromide for band visualization; added to the gel

prior to electrophoresis at a concentration of 0.05µl/mL. The gel was visualized with the Bio-Rad Gel Doc 1000 and the Quantity One 1-D Analysis Software (version 4.6.2 Basic). The presence of 2 clear bands (28S and 18S) and an absence of a smear ending in a low molecular-weight mark, verified the RNA was not degraded.

RNA was quantified spectrophotometrically (NanoVue, GE) and all samples were diluted to the same concentration with RNase-free water (between 0.7ug/uL - 1.0ug/uL). All samples were treated with RQ1 RNase-Free DNase (Promega) to eliminate possible DNA contamination as per the manufacturer's instructions.

2.4. Semi-quantitative RT-PCR

Two-Step RT-PCR was performed on all samples.

cDNA Synthesis. For all experiments, cDNA was generated using the DyNAmo cDNA Synthesis Kit (Finnzymes). The conditions were: 10 minutes at 25°C, 45 minutes at 37°C followed by 5 minutes at 85°C.

RT-PCR. Primers (Table 1) were designed for *Mad1, Mad2, Hsp30,* and *Hsp70* using Primer Select DNAStar Lasergene software based on the GenBank mRNA sequences (NCBI, GenBank). Published primers for *ssgA* and 18S rRNA were used (Fang and Bidochka 2006). The specificity of the primers was confirmed by a BLAST search in the nucleotide database (NCBI, nucleotide BLAST (blastn)) and used at final concentration of 0.5 µM in a 25 µL PCR reaction. The remaining reagents for PCR were supplied by New England BioLabs (NEB; Pickering, Ontario) and the conditions were as follows: initial denaturation at 95°C for 1 minute, 25

amplification cycles of 95°C for 20 seconds, primer temperature-specific annealing for 45 seconds, 72°C for 1 minute, and a final extension time of 72°C for 3 minutes.

Amplification of transcripts for *Mad*1 and *Mad*2 were performed in the same reaction tube while a second reaction tube contained primers for 18S rRNA. Amplification of *Mad*2, *Hsp*30, *Hsp*70 and *ssg*A transcripts in the stress test experiments were done separately from 18S rRNA due to similar amplicon size and required 35 amplification cycles. The 18S rRNA was used as the control for RNA titre and constitutive expression since it does not show significant changes (Fang and Bidochka 2006). A 5 μL aliquot of the resultant sample was loaded onto a 2% agarose gel, run for 60 minutes at 60V and semi-quantitative analysis to compare transcript abundance was performed using the software Quantity One (BioRad).

Statistical Analyses. All experiments were repeated three times and the resulting means were analyzed for overall significant differences by running a one-way ANOVA with a 5% level of significance ($\alpha = 0.05$) using AnalystSoft Inc., StatPlus:mac – statistical analysis program for Mac OS. Version 2009. (See www.analystsoft.com/en/). When evaluating the difference between two means, a student t-test for independent samples ($\alpha = 0.05$) was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Table 1. Primer Sequences for RT-PCR

Gene: Function	Sequence (5' – 3')	Ta, °C	Amplicon, bp
Mad2: Plant Adhesin	(for) GCC TCG TCT CCC GCA GTT GTC GTT CTC (rev) CCC CCT CCC CCT CCT CTC CAG TTT TAC AC	72	370
Mad1: Insect Adhesin	(for) AGA CTC CCC CTT GCC CTC CTG TT (rev) GTC TCG GCA CCG GTG GCA ATG A	67	247
18S rRNA: Ribosomal RNA (constitutive)	(for) AGG CCC GGG TAA TCT TGT (rev) GAC CTT GTT ACG ACT TTT ACT TCC TCT	60	266
*Hsp*70: 70kDa Heat Shock Response protein	(for) CCT CTC TCT CTT GGT ATT GA (rev) TTG TCG GAG AAA GTA GAG AA	53	139
*Hsp*30: 30kDa Heat Shock Response protein	(for) CCT TGC AAT AGA ACA TCC TT (rev) TAT TGT AAA AGG TTC GTG GG	53	121
ssgA: Starvation Stress Response	(for) TCC CAT CGA TGT GTA AAT TC (rev) CTC CTT TGT ACT TTT CTC CC	54	131

Ta: Annealing Temperature

2.5. *Mad2*-GFP Transformant

An in-depth description of how the *Mad2*-GFP construct was made and how *Metarhizium robertsii* was subsequently transformed can be found in Appendix D. A brief description of the processes involved is described below.

The open reading frame (ORF) of an engineered form of the green fluorescent protein (eGFP) from *Aequorea victoria* was inserted in place of the ORF of the *Mad2* gene in *M. robertsii* by the method of double-joint PCR (Yu et al. 2004) using the primers found in Table I of Appendix D. This construct was ligated into the plasmid pBARGEM7-2 (Pall and Brunelli 1993; obtained from the Fungal Genetics Stock Center, Missouri) and replicated in *E. coli* (JM109, New England Biolabs). Plasmids were purified using the GenElute Plasmid mini prep kit (Sigma) and *M. robertsii* was transformed by the protoplast method (Goettel et al. 1990; Wang and St. Leger 2006). Putative transformants were selected based upon glufosinate ammonium (GA) resistance when grown on minimal media M-100 agar plates containing 160 ug/uL GA. Transformants were confirmed for gene replacement by DNA extraction (Qiagen) and PCR amplification using the primers Mad2-Pro-U with EGFP-L and EGFP-U with Mad2-Term-L (Table I of Appendix D). The stability of the transformed culture was verified by subculturing five times on PDA plates.

A 500 mL Erlenmeyer flask containing 200 mL of YPD was inoculated with conidia harvested from a PDA culture of the *Mad2*-GFP transformant as previously described. After growing for four days in YPD the mycelium was filter-collected and washed three times in sterile, distilled water. 50 mL of Sabouraud dextrose broth (SDB), minimal media M-100, and three concentrations of bean root exudate (0.1%,

1% and 10% v/v) were inoculated with 1.0 g wet weight of fungal mass and incubated at room temperature for 12 hours on a rotary shaker (120 rpm). Fluorescence microscopy was performed using a Leitz Diaplan microscope equipped with light filters suitable for blue light excitation of eGFP (Leitz I2/3; excitation filter: 450–490nm, dichroic mirror: 510nm, emission filter: 515nm).

2.6. *Mad2* Promoter Analysis

The GenBank sequence for the complete *Mad2* gene (accession number: DQ338439.1) was input into the ALGGEN PROMO server (Messeguer et al. 2002; Farré et al. 2003) that utilizes version 8.3 of TRANSFAC, a transcription factor binding-site database. ALGGEN PROMO creates weight matrices for transcription factor binding site prediction based upon the information stored within TRANSFAC. The search was limited to transcription factors/binding sites from fungi, and to between the start of the sequence and 100 base pairs downstream of the translation start site (1080 base pairs total), as this is the common place of transcription initiation elements (Kornberg et al. 1994).

3. Results

3.1. *Mad1* and *Mad2* are Differentially Expressed in Media Containing Insect Cuticle or Bean Root Exudate.

The RT-PCR products of *Mad1* and *Mad2* were compared to that of 18S rRNA. The averages of all trials for the relative expression values are represented in Figure 2. *Mad1* was differentially up regulated in comparison to *Mad2* in locust and tobacco hornworm cuticle as expected from previous literature results (Wang and St. Leger 2007). Unexpectedly, cockroach cuticle had no significant difference between *Mad* gene expression (t-test, $p>0.05$). *Mad2* expression was significantly higher than *Mad1* expression in all concentrations of bean root exudate (0.1%, 1%, 10%), and with chitin and tomato stems. The expression of *Mad2* between each of the three concentrations of bean root exudate showed a significant difference (ANOVA, $p = 0.02$). *Metarhizium* grown in suspensions of cellulose and starch showed no statistical differences between *Mad1* and *Mad2* expression (t-test, $p>0.05$).

3.2. Differential Expression of *Mad* Genes in Exudates from Healthy and Damaged Roots.

Mad2 expression was significantly greater than *Mad1* when *Metarhizium* was grown in asparagus, carrot or basil exudate (t-test, $p<0.01$) (Figure 3). The expression of *Mad2* compared between healthy and damaged root exudate of the same plant species was not significantly different (t-test, $p>0.05$), however, *Mad2* expression between basil and asparagus or carrot was significantly different (t-test, $p<0.01$).

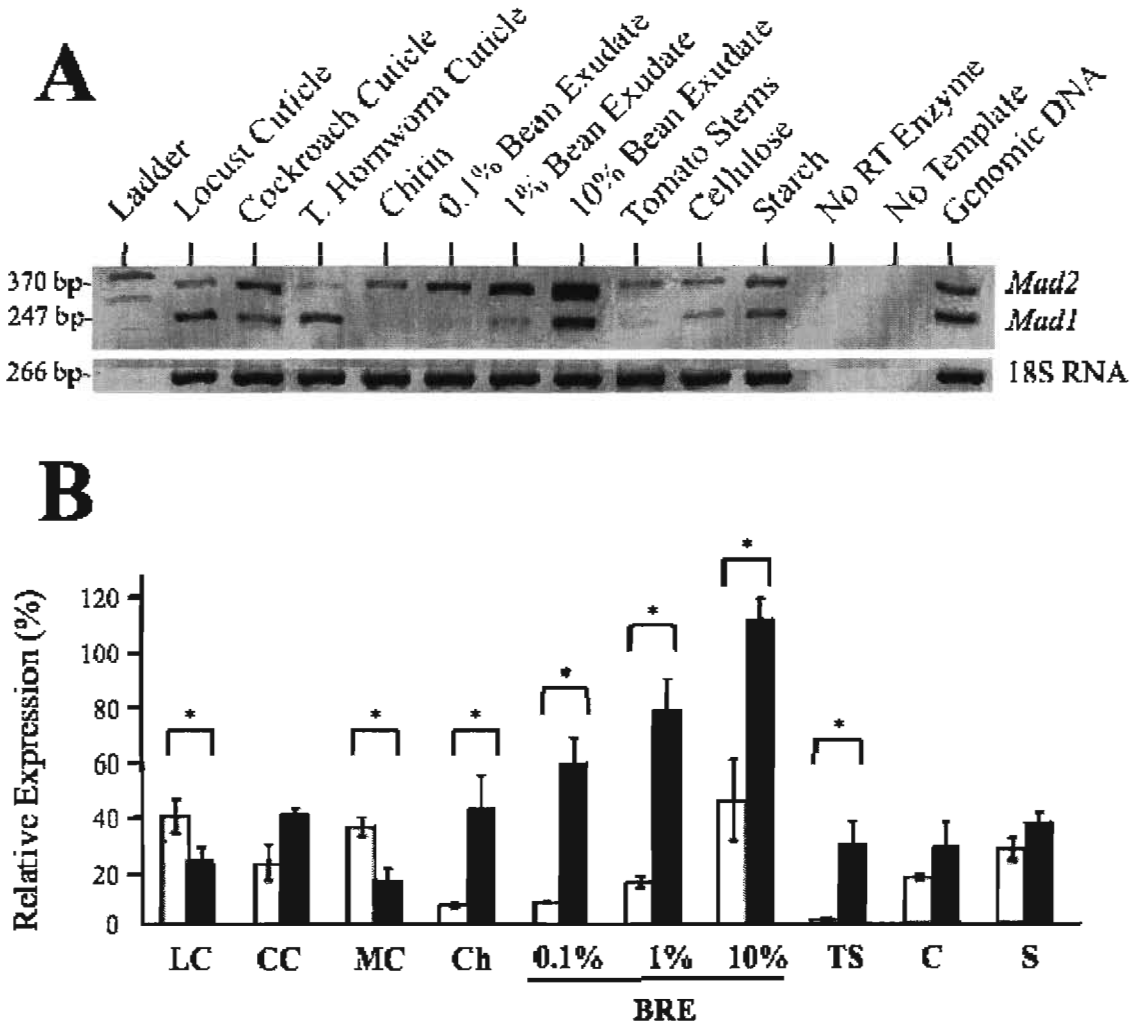

Figure 2. Relative gene expression of *Mad1* and *Mad2*. (A) RT-PCR analysis of *Mad1* and *Mad2* expression by *M. robertsii* ARSEF 2575 transferred from four-day grown YPD cultures to water-based 1% media, for 12 hours. Reference gene: 18S rRNA. (B) Bar graph quantifying the expression of *Mad1* (white bars) and *Mad2* (black bars) relative to 18S rRNA, derived from densitometric analysis of RT-PCR results. Values represent mean values ± standard deviations of three independent experiments. Asterisks denote a significant difference ($P < 0.05$). LC: locust cuticle; CC: cockroach cuticle; MC: tobacco hornworm cuticle; Ch: chitin; BRE: bean root exudate; TS: tomato stems; C: cellulose; S: starch.

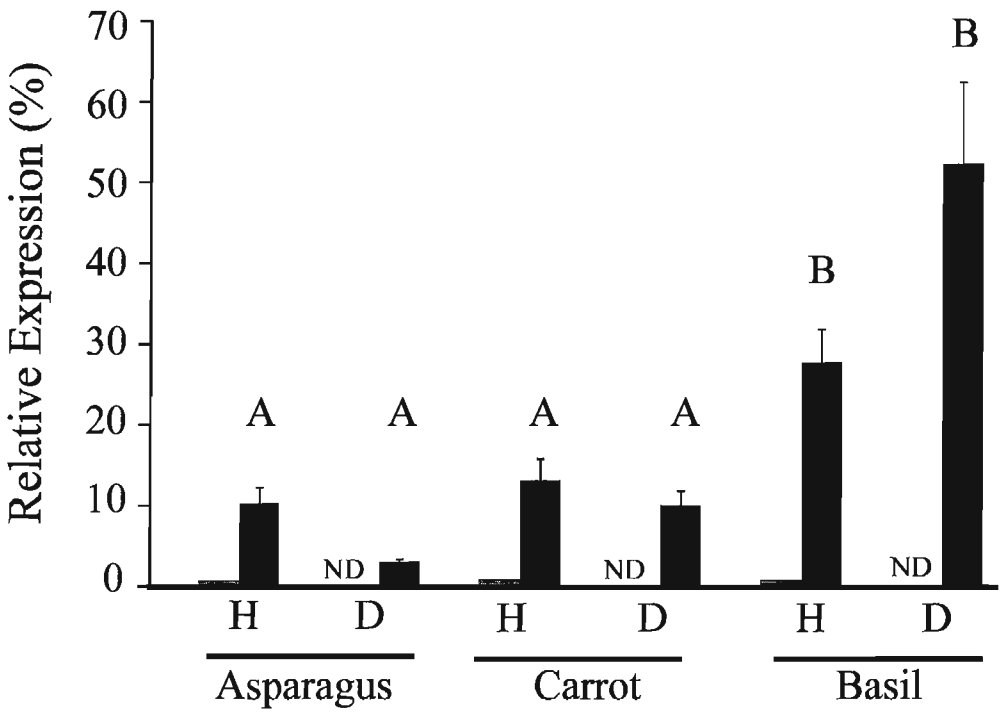

Figure 3. RT-PCR densitometric analysis of *Mad1* (grey bars) and *Mad2* (black bars) gene expression of *M. robertsii* ARSEF 2575 grown in water supplemented with 0.1 mg/mL of healthy- (H) and crushed- (D) root exudates, from seedlings of asparagus, carrot, and basil. Values are means of three independent experiments. *Mad2* graph bars with the same letter are not significantly different ($P > 0.05$). ND: not detected.

3.3. Carbohydrate Preference Affects *Mad2* Expression.

Figure 4 shows the expression of *Mad2* when *Metarhizium robertsii* was grown for 12 hours in 1% carbohydrate solutions with and without the addition of 0.1% peptone. *Mad2* expression significantly decreased (t-test, $p<0.05$) with the addition of peptone (a source of both carbon and nitrogen) for trehalose, raffinose, lactose, sorbose and fructose. With the exception of trehalose, these carbohydrates are classified as non-preferred carbohydrate sources for this strain of *Metarhizium* (Rangel et al. 2006; Rath et al. 1995). The preferential sugars, glucose and maltose, showed no significant difference in *Mad2* expression with the addition of peptone (t-test, $p>0.05$). Glucose, the most readily metabolized sugar of fungi (Griffin 1981), and maltose, a disaccharide composed of two molecules of glucose, resulted in consistently low expression of *Mad2* regardless of the addition of peptone. The amount of *Mad2* transcripts was barely detectable in the solution of NAG without peptone but significantly increased (t-test, $p<0.05$) with the addition of peptone. Arabinose showed no significant change in *Mad2* expression (t-test, $p>0.05$) with the addition of peptone and the expression level was equivalent to that of the non-preferred sugars without peptone.

The expression of *Mad1* was also analyzed but there was no significant difference between *Mad1* and *Mad2* gene expression in the 1% carbohydrate media, with the exception of sorbose (Table 6-7, Appendix C). With the addition of peptone, *Mad1* gene expression was significantly higher than *Mad2* for all carbohydrates, with the exception of lactose that was significantly lower (Table 8-9, Appendix C). There was no discernible pattern for the change in *Mad1* gene expression between *M.*

robertsii grown in 1% carbohydrate compared to the same carbohydrate with the addition of 0.1% peptone (Table 10, Appendix C).

3.4. *Mad2* is Starvation-Stress Induced.

The relative expression of *Mad2* over the course of 72 hours in sterile-distilled water, minimal media (M-100) and YPD is shown in Figure 5. *Mad2* expression in water increased significantly from 15 minutes to 2 hours and from 2 to 8 hours (t-test, $p<0.05$). From 12 to 24 hours, there was a significant increase (t-test, $p<0.01$) in *Mad2* expression equivalent to that at 8 hours. After 24 hours the expression continued to decline significantly between each remaining time interval (t-test, $p<0.01$) (Figure 5A).

The relative expression of *Mad1* was barely detectable for all time points, significantly less than the expression of *Mad2* for all but 0.25 hours (Table 11, Appendix C).

A trend of increased *Mad2* expression over time as the length of starvation increased was present in the time course analysis of minimal media M-100 as well (Figure 5B). There was a significant difference between the time intervals 0 to 2 hours (t-test, $p<0.05$), 2 to 4 hours (t-test, $p<0.001$) and 8 to 12 hours (t-test, $p<0.05$). From 12 to 72 hours *Mad2* expression did not significantly change (t-test, $p>0.05$).

The expression of *Mad1* was detectable up to and including 24 hours but did not significantly differ from the expression of *Mad2* for the time intervals of 0.25, 2, and 8 hours. For all other time points, the expression of *Mad1* was differentially less than that of *Mad2* (Table 12, Appendix C).

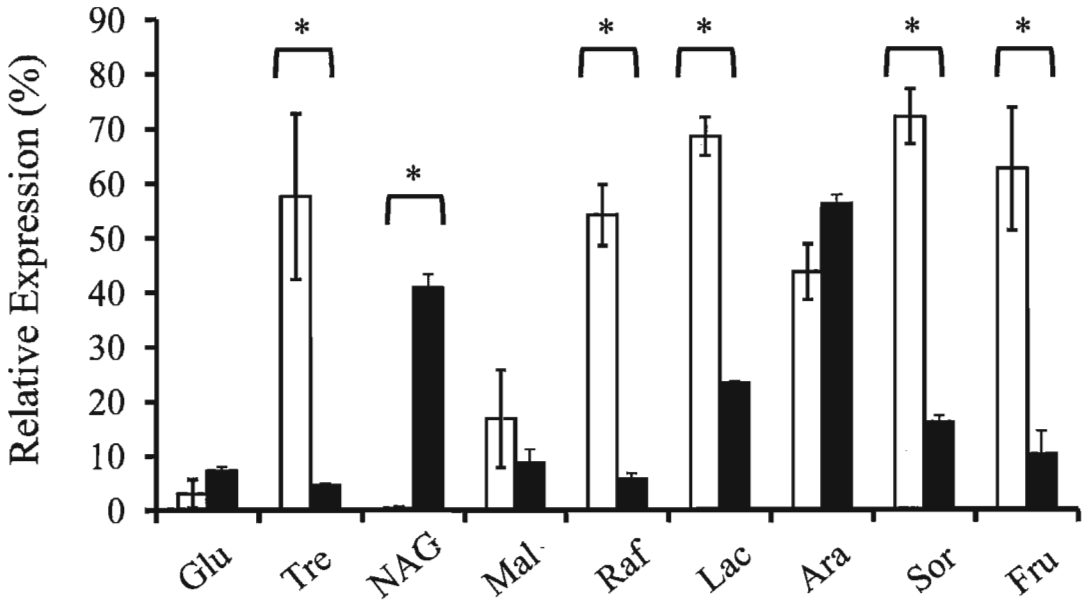

Figure 4. Relative gene expression of *Mad2*. Graph bars quantifying the expression of *Mad2* relative to 18S rRNA, from RT-PCR densitometric analysis of *M. robertsii* ARSEF 2575 grown for 12 hours in 1% carbohydrate media (white bars) and 1% carbohydrate with 0.1% peptone media (black bars). Values represent mean values ± standard deviation of three independent experiments. Asterisks denote a significant difference ($P < 0.05$). Glu: glucose; Tre: trehalose; NAG: N-acetylglucosamine; Mal: maltose; Raf: raffinose; Lac: lactose; Ara: arabinose; Sor: sorbose; Fru: fructose.

The time course analysis of YPD yielded significant differences in *Mad2* expression between every time interval (t-test, $p<0.05$) except between 8 to12 hours and 48 to 72 hours (t-test, $p>0.05$) (Figure 5C). *Mad2* expression increased from 2 hours to its peak at 8 hours and then continually declined up to 72 hours where it was not detected (Figure 5C). At time zero in YPD, *Mad2* was expressed more than at time zero in water and M-100 and more than at 2 hours in YPD. This expression of *Mad2* is an artifact due to using the four-day old culture of *Metarhizium* and neglecting to transfer it to fresh YPD medium for 15 minutes. It did however highlight the immediate effect of fresh media (decrease in expression between 15 min. in YPD and 15 min. sterile water) and was the first instance within this study of the level of *Mad2* transcription of the inoculum after germination and growth for four days in YPD.

The expression of *Mad1* mimicked the pattern of *Mad2* expression for all time points (Table 13, Appendix C).

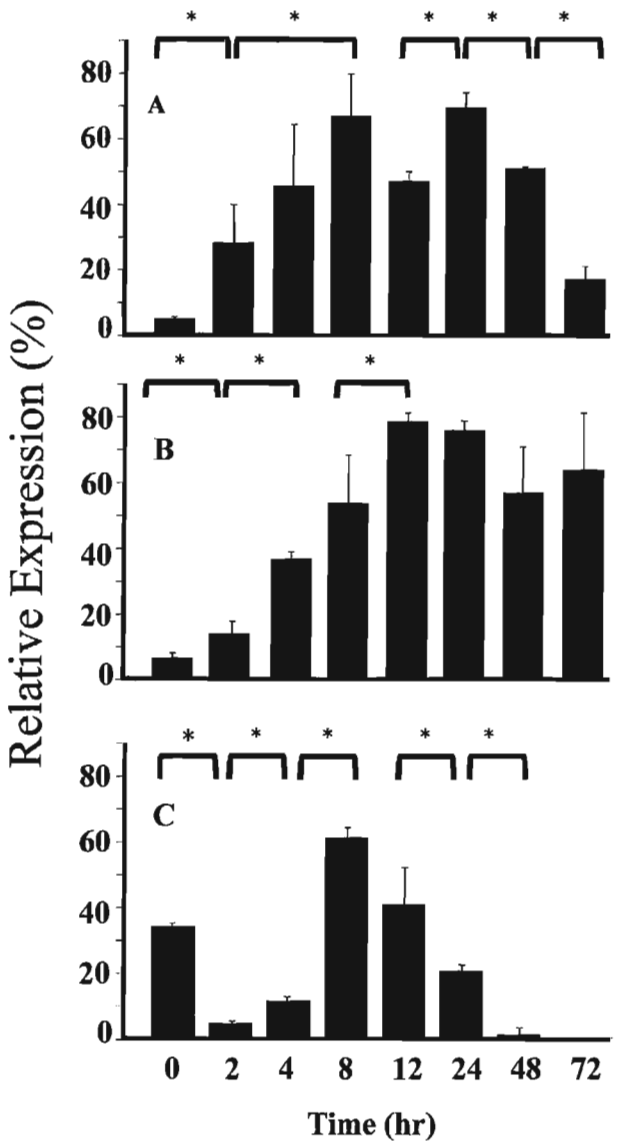

Figure 5. Time-course analysis for *Mad2* expression. Densitometric RT-PCR analysis of the expression of *Mad2* of *M. robertsii* ARSEF 2575 transferred from four-day grown YPD cultures to (A) sterile-distilled water, (B) minimal media M-100, and (C) YPD, for 0 to 72 hours. Each time interval is an independent sample. Reference gene: 18S rRNA. Graph bars represent mean values ± standard deviation of three independent experiments. Asterisks denote a significant difference ($P < 0.05$).

3.5. *Mad2*-GFP Transformant Shows Highest Fluorescence in Minimal Media.

The transformed culture of *M. robertsii* that had GFP expression under the control of the *Mad2* promoter had fluorescence for all media tested (Figure 6). This was expected as previously literature had detected *Mad2* transcripts in all media tested thus far (Wang and St. Leger 2007). The nutrient rich broth SDB had the lowest GFP fluorescence whereas minimal media had the highest fluorescence. The fluorescence of the bean root exudate media was higher than in the SDB media but lower than the minimal media. The transformant fluorescence profiles support the preceding evidence of nutrient deprivation induction of *Mad2*.

3.6. *Mad2* Expression is Starvation Induced Independent of pH, Heat, Osmotic or Oxidative Stress.

Figure 7 compares the expression of *Mad2* to a known nutrient stress response gene, *ssgA* (starvation stress gene A), and the 30kDa and 70kDa heat shock protein (*Hsp*) genes relative to the expression of 18S rRNA. The various stresses initially tested were temperature (15°C and 37°C) pH (4 and 10), osmotic (0.7M KCl and 0.5M sorbitol), oxidative (10mM H_2O_2 and 100mM H_2O_2) and starvation stress. Starvation stress was evaluated by comparing the results of YPD-based and water-based media.

There was no significant difference (t-test, $p>0.05$) between each concentration of KCl, sorbitol and H_2O_2 for the expression of any of the genes tested within the same media (Table 20). Therefore, of all stress tests performed, only pH 4,

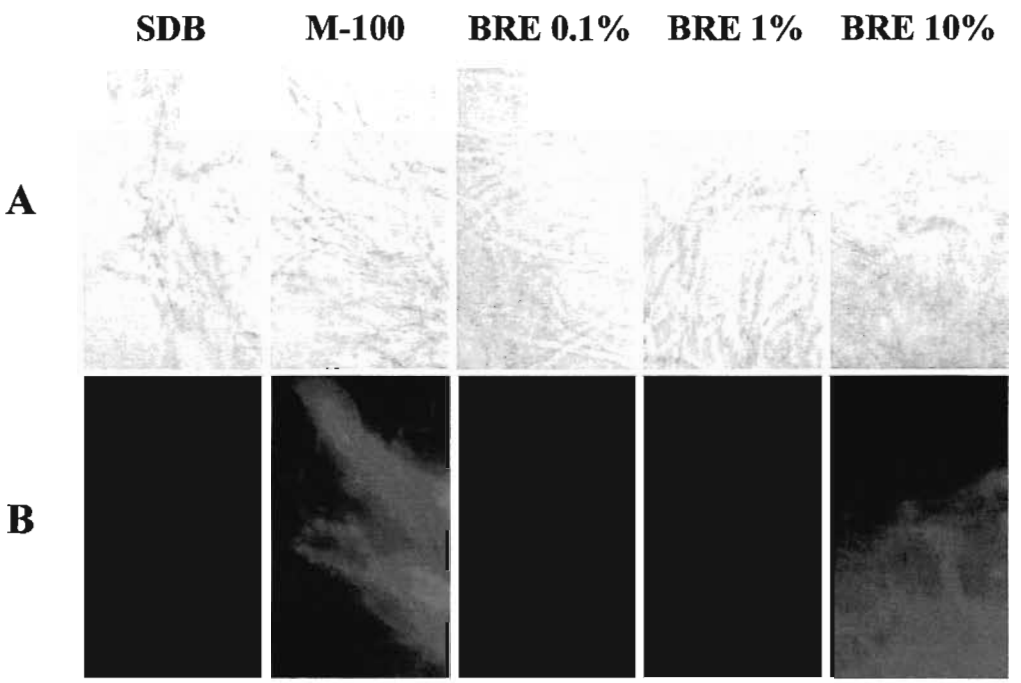

Figure 6. *Mad2*-GFP transformant fluorescence microscopy. *M. robertsii* 2575 transformant expressing GFP under the control of the *Mad2* promoter (*Mad2*-GFP transformant) grown for 12 hours in Sabauroud dextrose broth (SDB), minimal media (M-100) and bean root exudate (BRE) at room temperature. (A) Light Microscopy, (B) Fluorescence Microscopy. Magnification = 40X. Exposure time = 6 seconds.

pH 10, 37°C, 0.7M KCl and 10mM H_2O_2 for each media type are shown in Figure 7 as they are representative of each type of stress.

In the nutrient rich medium YPD, *ssgA* transcripts were not detected in any of the test conditions whereas in the water-based media *ssgA* transcripts were detected to varying degrees under all conditions (Figure 7; Table 17, Appendix C). Similarly, *Mad2* was detected at very low amounts in all YPD-based media, insignificantly different from each other (ANOVA, $p>0.05$). Within the water-based media, *Mad2* expression was dramatically higher than YPD in all conditions but the level of expression was the same (Figure 7; Table 16, Appendix C). The expression of *Mad2* not significantly different among the various stress conditions and barely detectable in any condition under nutrient rich conditions indicates that the effect was due solely to nutrient deprivation rather than pH, temperature, oxidative or osmotic stress.

The expression of the heat-inducible *Hsp*70 was highest at the elevated temperature of 37°C, significantly different from any other stress conditions in both YPD-based and water-based media (ANOVA, $p<0.05$) (Figure 7; Table 19, Appendix C). The expression of *Hsp*70 was significantly higher in all water-based stress conditions in comparison to the YPD counterparts (t-test, $p<0.05$) confirming the effect of nutrient starvation, as it is another inducer of *Hsp*70 transcription (Lindquist and Craig 1988; Ruis and Schüller 1995).

The same starvation trend was seen with the expression of *Hsp*30. In the YPD-based media, all conditions were significantly lower than the expression levels in the water-based media (t-test, $p<0.05$). In both the YPD- and water-based media

37°C was the condition in which *Hsp*30 was more up regulated than all the other stress conditions (ANOVA, $p<0.05$).

To confirm that this effect seen was starvation stress and not due to a possible inherent effect of osmotic stress from the water being distilled, the expression of *Mad2* was evaluated in a sterile phosphate buffered saline (PBS) solution compared to sterile-distilled water. There was no significant difference between the expression of *Mad2, ssgA, Hsp*30 or *Hsp*70 in PBS compared to distilled water (Figure 9-10, Appendix A).

3.7. The *Mad2* Promoter has Two Putative STRE Transcriptional Elements.

Examination of the *Mad2* promoter using the ALGGEN server (Messeguer et al. 2002; Farré et al. 2003) revealed putative transcription factor (TF) binding sites. Table 2 lists the TFs that had the highest core:matrix match found within the sequence up to and including the 5' UTR (untranslated region). Although the presence of one sequence does not always result in functionality, the occurrence of more than one is more indicative of it. As the majority of results were related to gene regulation by nutrient availability, taken together with the results obtained through the stress tests and time-course analysis, many of these transcriptional elements are likely functional and regulate *Mad2* gene expression.

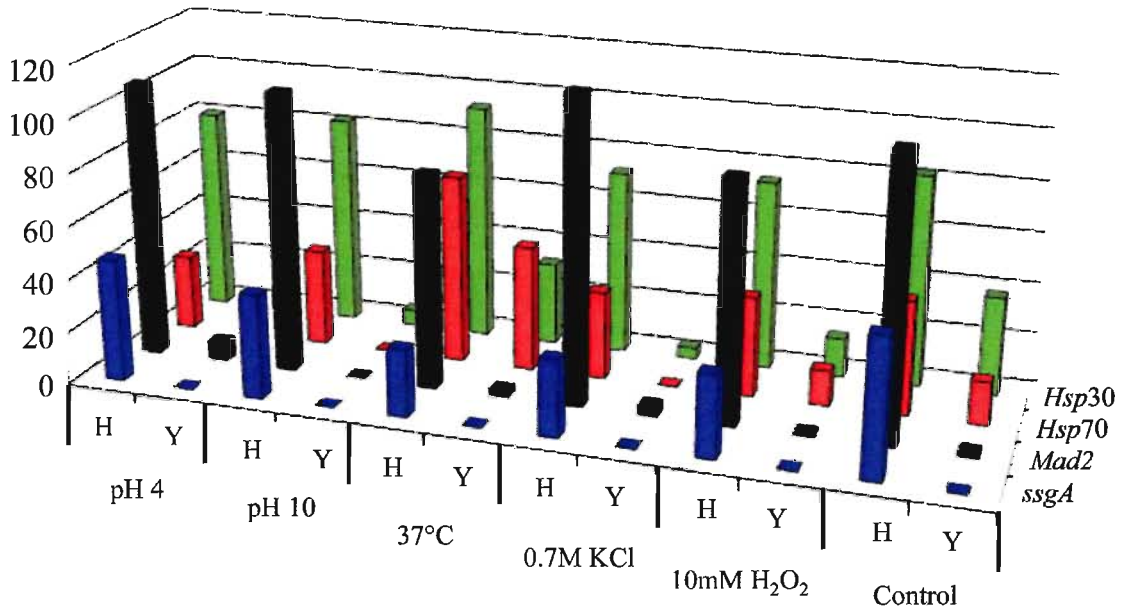

Figure 7. Effect of stress on the expression of *Mad2* and stress-related genes. RT-PCR densitometric analysis of *M. robertsii* ARSEF 2575 grown in YPD (Y) and sterile-distilled water (H) media, with listed stress conditions: media adjusted to pH 4 (with 1M HCl) or pH 10 (with 1M NaOH); incubated at 37°C; supplemented with 0.7M KCl or 10mM H_2O_2. Control is unadjusted media incubated at room temperature. Samples were incubated for 2 hours. Graph bars represent mean values of three independent experiments relative to 18S rRNA. Refer to Tables 16-23 (Appendix C) for data and statistical significance tests.

Table 2. Putative transcriptional elements within the promoter region of *Mad2* of *M. robertsii* 2575 detected using PROMO (version 8.3 TRANSFAC) of the ALGGEN server (Messeguer et al. 2002; Farré et al. 2003). Gene location is upstream of the translation start site.

Transcription Factor	Role	Core match : Matrix match	Upstream Location
HSF	Heat Shock Factor: gene activator during heat shock, glucose limitation, weak organic acid stress, and osmotic stress.[1]	1.000 : 1.000	-202
ADR1	Alcohol Dehydrogenase Regulator 1: activator of genes regulated by glucose repression.[2,3]	1.000 : 0.997	-331
Dde box	Related to transcriptional efficiency and corresponds to region responsible for carbon regulation.[4]	1.000 : 0.992	-508
REB1	RNA Pol I Enhancer Binding Protein: binds genes transcribed by Pol I and Pol II.[5]	1.000 : 0.988	-30
STRE	Stress Response Element: activates genes regulated by heat shock, nitrogen/glucose starvation, osmotic/oxidative/weak acid/ethanol/pH stress.[6,7]	1.000 : 0.972 1.000 : 0.974	-949 -919
GCN4	General Control Nondepressible 4: activates genes expressed during amino acid starvation.[8,9]	1.000 : 0.949	-283
Repressor of CAR1	Represses transcription of arginase (CAR1) which is under arginine and nitrogen catabolite repression.[10,11]	1.000 : 0.937	-948
FACB	Factor of Acetate B: activator of acetate utilization genes during glucose limitation.[12]	1.000 : 0.804	-801
MIG1	Multicopy Inhibitor of GAL gene: negatively regulates genes under glucose catabolite repression.[13,14]	1.000 : 0.849	-747
HAP2/3/4	Subunit of glucose repression CCAAT-binding complex.[15]	0.891 : 0.871	-282
PDS	Post Diauxic Shift element (T/AAGGGA): mediates transcriptional activation in response to nutritional limitation.[16,17]	NA – self identified	-109
BRE	brlA response element (C/AG/AAGGGG/A): Regulator of asexual development in *Aspergillus*, necessary for conidiophore development in response to starvation and osmotic stress.[18, 19]	NA – self identified	-236

[1]Wu 1995; [2]Ciriacy 1975; [3]Young et al. 2003; [4]Tyler et al. 1991; [5]Badis et al. 2008; [6]Marchler et al. 1993; [7]Gorner et al. 1998; [8]Fernandes et al. 1997; [9]Natarajan et al. 2001; [10]Coffman et al. 1996; [11]Messenguy et al. 2000; [12]Todd et al. 1998; [13]Carlson 1999;

[14]Schuller 2003; [15]Gancedo 1998; [16]Boorstein and Craig 1990; [17]Pedruzzi et al. 2000; [18]Chang and Timberlake 1993; [19]Adams and Wieser 1999.

4. Discussion

4.1. The Expression of *Mad2* is Induced by Starvation

Analysis of differential gene expression gives insight into how *Metarhizium robertsii* perceives and responds to its immediate environment. *M. robertsii* has a bifunctional lifestyle and up regulates genes associated with insect pathogenesis or plant symbiosis. This is exemplified by the up-regulation of *Mad1* in response to insect cuticle/hemolymph in comparison to the up regulation of *Mad2* with exposure to bean root exudate (Wang and St. Leger 2007). The goal of this study was to further investigate the regulation of *Mad2* gene expression.

There was a significant difference in *Mad2* gene expression with regards to the concentration of bean root exudate. The positive correlation between *Mad2* gene expression and bean root exudate suggests that *Metarhizium* can perceive and respond to plant root compounds. Previous literature has shown that high concentrations of bean root exudate negatively impact germination of *M. robertsii* conidia (Fang and St. Leger 2010) and that expression of a putative ABC-transporter, well known for the removal of toxic substances from a cell, is up regulated (Pava-Ripoll et al. 2011). The increase in *Mad2* expression with higher concentrations of bean root exudate may therefore be linked to a stress response element.

The finding in field experiments that GFP-expressing *Metarhizium* associated with plant roots over a longer period than in bulk soil (Hu and St. Leger 2002; Fang and St. Leger 2010) suggests a chemotactic ability to locate and associate with plant roots. In other rhizospheric symbioses, plants stimulate the attraction of a beneficial fungal partner by releasing "branching factors" into their exudate that cause an increase in fungal

branching; increasing probability of root contact (Bais et al. 2006). The fungus moves through the concentration gradient that develops around the plant roots, the highest concentration being closest to the rhizoplane (root surface) (Uren 2007). As hyphae of *M. robertsii* explore the soil, and sense the increased concentration of plant compounds, transcription of *Mad2* may increase in preparation for fungal attachment to the root. Once *M. robertsii* has penetrated the plant cuticle (Sasan and Bidochka, personal communication; Liu et al. 2009) and starts to grow within the nutrient-rich (i.e. glucose) cortical cells/interstitial space (Hancock 1977), the expression of *Mad2* would be repressed. This suggests that *Mad2* is regulated by nutritive stress.

Growth of *M. robertsii* on non-preferred carbon sources by Rangel et al. (2006) showed that *M. robertsii* was under nutritive stress by evidence of the development of cross-protection to UV-B. When we investigated the effects of various carbohydrates, *Mad2* expression was highest when *M. robertsii* was grown with a non-preferred carbohydrate as the sole carbon source (i.e. raffinose, sorbose, lactose, arabinose and fructose). Comparatively, when a preferred carbon source was present, such as glucose, maltose or NAG, *Mad2* expression was barely detectable, suggesting that *Mad2* is likely under carbon catabolite repression.

The exception within our results was the high expression of *Mad2* when trehalose was the sole carbon source. Trehalose is the most abundant carbohydrate in the hemolymph of insects as its hydrolysis yields two molecules of glucose readily used for flight initiation (Xia et al. 2002; Becher et al., 1996). Locusts infected with *Metarhizium* have a rapid decrease in trehalose content coincident with a transient increase in transcription of a neutral trehalase (extracellular trehalose-hydrolyzing enzyme) by

Metarhizium (Zhao et al. 2006). Additionally there have been reports of *Metarhizium* being able to grow on a basal medium with trehalose as the sole carbon source (Campbell et al. 1983; Rangel et al. 2008). Our results seemingly contradict all of our other data that supports *Mad2* expression is repressed by the presence of a preferred carbohydrate (i.e. glucose, NAG, and maltose – also a disaccharide of glucose). However, trehalose may be an exception as it is a very diverse carbohydrate whose role is not simply nutritive.

Studies that showed the ability of *Metarhizium* to utilize trehalose as a sole carbon source utilized minimal media that contained salts and vitamins (Rangel et al. 2008; Campbell et al. 1983). In this study, there was only the carbohydrate of interest and distilled water as the medium for growth. Trehalose is accumulated in response to nitrogen starvation (Uyar et al. 2010; Rangel et al. 2008) and would thus not be accessible for metabolism until alleviation of that stress. This explains why the level of *Mad2* expression in trehalose alone was equivalent to that of the non-preferred carbohydrates and was barely detectable (as with other preferred carbohydrates) when peptone – a carbon and nitrogen source, was added in addition to trehalose.

Plant root exudates contain a variety of carbohydrates, but similarly they primarily contain glucose, fructose, xylose, arabinose and sucrose (Buxton 1962; Rovira 1965; Rovira 1969; Jalali and Suryanarayana 1971; Vancura and Stanek 1975). The amount of a solute exuded by the plant root compared to the amount within the intercellular space differs vastly. Hancock (1977) measured the approximate concentration of glucose in the intercellular space of squash root cells to be between 2mM and 6mM. This concentration is several folds higher than the approximate concentration of glucose found in root exudates (ca. 0.033mM) (Rovira 1969; Jalali and

Suryanarayana 1971; Vancura and Stanek 1975). Therefore, compared to the intercellular compartment of roots, the rhizosphere is a relatively nutritionally depleted niche. Repression of *Mad2* would not occur until *Metarhizium* had penetrated the root cuticle and began to grown within the glucose-rich intercellular space. Other carbohydrates could act as plant root signal molecules. For example, in the arbuscular mycorrhizal fungus, *Glomus intraradices*, hyphal growth was stimulated in response to raffinose (Hildebrandt et al. 2006).

Root exudates vary based upon plant species/age, temperature, light and nutrient status (Rovira 1969; Czarnota et al. 2003). By varying the composition of exudate, plant species can stimulate/inhibit various organisms, thus coordinating colonization of the rhizosphere with preferential microorganisms. The exudate of basil, compared to asparagus and carrot exudate, resulted in higher *Mad2* expression indicating the differential effect of plant type, and thus plant exudate, on *Mad2* expression. The exclusive association of different species of *Metarhizium* with different plant species in Ontario, Canada (Wyrebek et al. 2011; Bidochka et al. 1998) supports this idea and may be mediated through regulation of *Mad2*. Whether or not MAD2 is involved in plant recognition or if its expression is solely a consequence of root exudate composition remains to be determined

Expression of *Mad2* was linked with nutrient starvation. As time of starvation increased, there was a respective increase in *Mad2* expression. A diauxic shift is the preferential utilization and exhaustion of one carbon source, followed by a lag in growth as the enzymes required for digestion of the second available carbon source are made, after which growth resumes (Jones and Kompala 1999). The expression of *Mad2* from

time zero (four-day old inoculum) to 2 hours in fresh YPD significantly decreased. At eight hours there was maximal expression of *Mad2* followed by a continual decline in expression, possibly representing a shift in metabolism. *M. robertsii* was shown to possess and express enzymes of the glyoxylate shunt, a bypass for gluconeogenesis when glycolysis is insufficient (Padilla et al. 2011). When *M. robertsii* was grown in 1% and 10% bean root exudate, the gene encoding isocitrate lyase (converts isocitrate to glyoxylate) was found to be expressed (Padilla et al. 2011), similarly *Mad2* was expressed under these conditions. The activation of the glyoxylate cycle, as well as the up regulation of *Mad2*, gives insight into the metabolic activities of *M. robertsii* within the rhizosphere and supports the nutrient limitations of bean root exudate.

A time course analysis that examined *Mad1* and *Mad2* gene expression *in vivo* of *Plutella xylostella* infected by *M. robertsii* (Barelli et al. *in press*) reinforced the impact of the availability of nutrients on the regulation of *Mad2*. During the late stages of infection, coincident with outgrowth and conidiation on the surface of the insect cadaver, expression of *Mad2* was up regulated. This pattern of gene expression is similar to that of the hydrophin, *ssgA* (starvation stress gene), and subtilisin-like protease, Pr1A, of *M. robertsii* (St. Leger et al. 1992; Small and Bidochka 2005). When *Metarhizium* was grown in nutrient rich media, transcripts of ssgA were low or not detected at all (St. Leger et al. 1992). St. Leger et al. (1992) showed that in comparison, growth in nutrient poor media showed a marked increase in *ssgA* expression that could be halted with the addition of glucose. This pattern of expression of *ssgA* was correlated with that of Pr1A, known to be highly responsive to nutrient deprivation (St. Leger et al. 1992; St. Leger et al. 1989). *In vivo* examination of Pr1A expression during infection of *Galleria mellonella*

showed that it was up regulated during early and late stages of infection (Small and Bidochka 2005). This protease is required to penetrate the insect cuticle and its expression is induced early in the infection to the lack of accessible nutrients of the epicuticle. Nutrient deprivation again induces Pr1A expression during late stages of infection when the *Metarhizium* escapes the insect cadaver and conidiates on the surface (Small and Bidochka 2005). This pattern of Pr1A expression during cadaver mummification is mimicked by the expression of *Mad2*, corroborating the role of nutrient deprivation in *Mad2* gene expression.

The expression of *Mad2* was up regulated by nutrient stress (similar to *ssgA*) independent of temperature, pH, and osmotic or oxidative stresses. In comparison, known stress-response genes *Hsp*30 and *Hsp*70, were up regulated under stress conditions when grown in nutrient rich media (i.e. YPD). Although the promoters of genes responsive to different stresses can share regulatory elements (i.e. STRE; a general stress responsive element), the context of the entire promoter will dictate when a gene is transcribed (Wu 1995; Estruch 2000). The promoter of *Mad2* contains several STRE sequences, whose transcription factors Msn2/Msn4 respond to numerous types of stress (Martinez-Pastor et al. 1996), but it mainly consists of elements that are related to glucose repression as explained earlier (see Table 2). The *in silico* context of *Mad2* expression is then primarily activation in response to limitation of an easily metabolized carbon source, supporting the experimental findings of this research.

Understanding the regulation of genes, such as *Mad2*, required for this entomopathogenic, beneficial root-colonizer to initiate and maintain a relationship with the roots of plants would help develop a global understanding of the attributes of the

rhizosphere. Identifying homologous proteins in other fungal species may reveal other potential biopesticides and may help to eliminate the need for chemical agents all together.

4.2. Tripartite Interactions within the Rhizosphere?

M. robertsii has not been shown to be pathogenic to any plant species (Zimmermann 2007) as is common to many plant-symbiotic members of the Ascomycota (Montanini et al. 2006, Nehls 2008). *M. robertsii* could serve as a beneficial partner for plants due to its entomopathogenic capabilities to a broad range of insects (Wang et al. 2004). The association with the plant rhizosphere could serve as a physical barrier against a soil-dwelling insect or act as an antagonist against a fungal pathogen. In other symbiotic fungal-plant associations such as mycorrhizal and endophytic-associations the fungal partner receives carbohydrate and lipid nutrition in exchange for delivering nitrogen and phosphorus to the host plant (Scervino et al. 2005). If a rhizospheric symbiosis exists with *M. robertsii* then this relationship may actually be tripartite. While *M. robertsii* parasitizes an insect, from which it liberates nutrients, it could subsequently share these nutrients with the host plant in exchange for continual supply of carbohydrates. An association of this type gives plants access to niches otherwise unattainable and the fungus receives a carbon supply much greater than the amount from simple root leakage of the plant (Nehls 2008).

Determining precise regulation of *Mad2* may help in manipulating a protective association between *M. robertsii* and the roots of economically important plants not typically colonized by this fungus.

5. Concluding Remarks

Understanding the regulation of the plant adhesin gene, *Mad2*, of *Metarhizium robertsii* will help us to better understand rhizosphere biology. The ability of *Metarhizium* to moderate a pathogenic lifestyle with insects and yet a symbiotic lifestyle with plants is remarkable. Coordination of this biphasic lifestyle may be determined by a better understanding of the regulation of the adhesin-encoding genes, as the adhesins are essential for initiating both lifestyles. This study determined that *Mad2* is regulated by nutrient starvation. The limited nutrient availability of both insect cuticle and root exudates reveals the existence of an unidentified substrate-specific stimulus the fungus recognizes in order to initiate differential gene expression. Future research should focus on determining the specific pathway(s) that regulate transcription of *Mad2*, the relationship between *Mad2* transcription and protein expression, and any specific binding activities of MAD2 to elucidate whether it plays a role in plant-host specificity. Isolating individual components of root exudate and analyzing the expression of *Mad2* under minimal nutrient conditions may elucidate a plant-specific molecule or compound that induces *Mad2* differentially from *Mad1,* that may in turn help in revealing an insect-specific inducer. The mechanism of adhesin biology is applicable across all areas of microbiology, impacting everything from agriculture and the economy, to medicine and human health.

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Zimmermann G, 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology* **17:** 879-920.

Appendix A:
Agarose Gel Images

The following are several representative agarose gels from selected experiments.

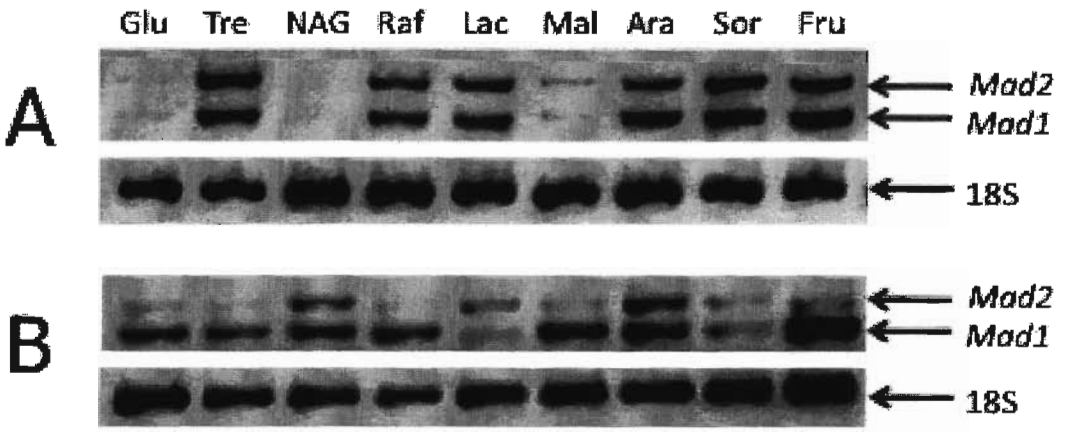

Figure 8. RT-PCR results for *Mad1*, *Mad2* and 18S expression when *M. robertsii* 2575 was grown for 12 hours at room temperature in sterile distilled water with (A) 1% carbohydrate or (B) 1% carbohydrate with 0.1% peptone. Three replicates performed, one representative of each is shown. Glu: glucose; Tre: trehalose; NAG: N-acetylglucosamine; Raf: raffinose; Lac: lactose; Mal maltose; Ara: arabinose; Sor: sorbose; Fru: fructose.

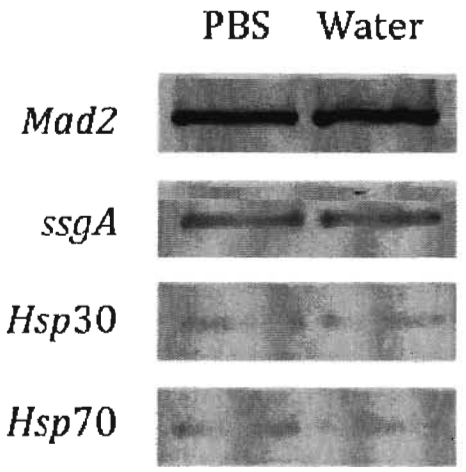

Figure 9. RT-PCR results of *Mad2*, *ssgA*, *Hsp*30 and *Hsp70* gene expression for *M. robertsii* 2575 grown in phosphate buffer saline (PBS) solution and sterile-distilled water.

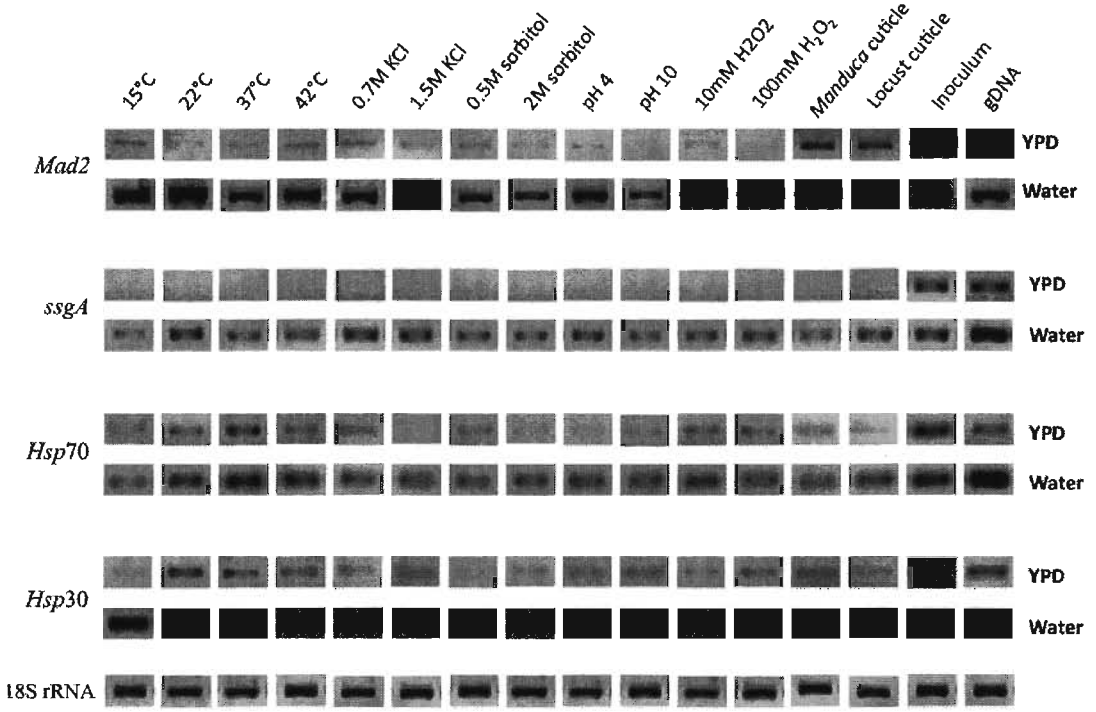

Figure 10. RT-PCR results for stress tests. Expression of *Mad2*, *ssgA*, *Hsp*30, *Hsp*70 and 18S rRNA (reference gene) for *M. robertsii* 2575 grown in YPD or sterile-distilled water under various stress conditions, for 2 hours at room temperature. Three replicates performed, one representative of each is shown.

Appendix B.
Mad1 Relative Expression Graphs

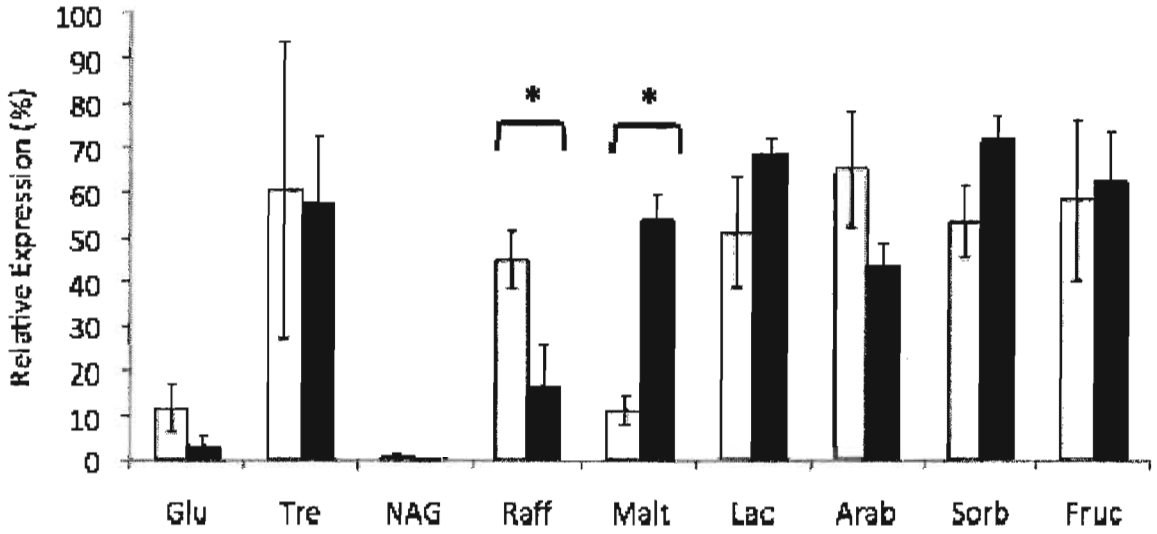

Figure 11. 1% carbohydrates on *Mad* genes. Relative expression of *Mad1* (white bars) and *Mad2* (black bars) for *Metarhizium* grown in 1% carbohydrate media for 12 hours at room temperature. Reference gene, 18S rRNA. Bars represent mean values ± standard deviation of three independent experiments. Asterisks denote a significant difference (t-test, $P < 0.05$).

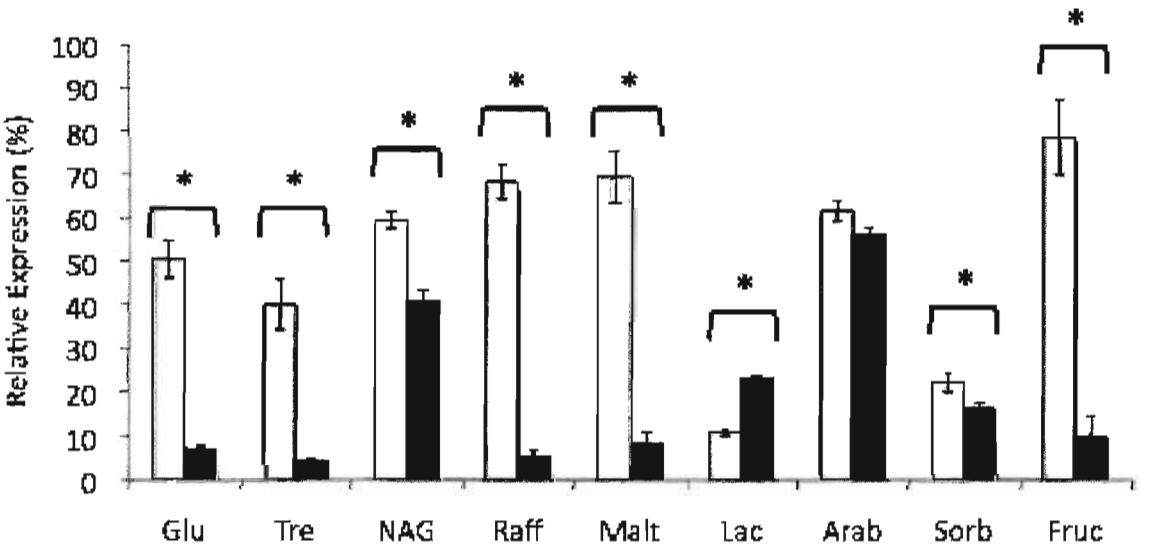

Figure 12. 1% carbohydrate + 0.1% peptone on *Mad* genes. Relative expression of *Mad1* (white bars) and *Mad2* (black bars) for *Metarhizium* grown in 1% carbohydrate media for 12 hours at room temperature. Reference gene, 18S rRNA. Bars represent mean values ± standard deviation of three independent experiments. Asterisk denotes a significant difference (t-test, $P < 0.05$).

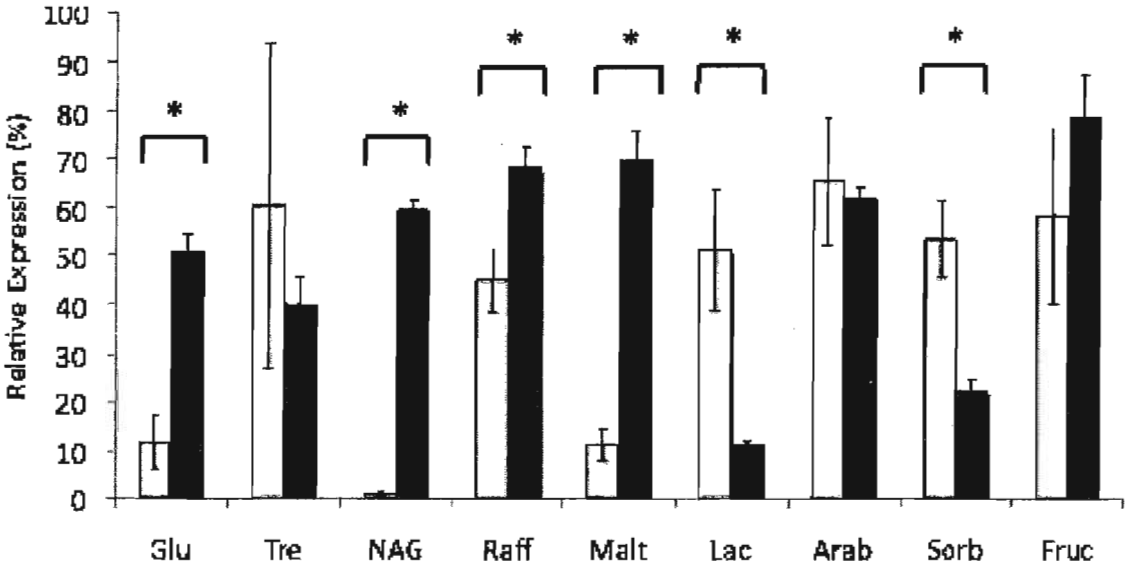

Figure 13. *Mad1* expression. Relative expression of *Mad1* for *Metarhizium* grown in 1% carbohydrate media (white bars) or 1% carbohydrate + 0.1% peptone (grey bars) media for 12 hours at room temperature. Reference gene, 18S rRNA. Bars represent mean values ± standard deviation of three independent experiments. Asterisk denotes a significant difference (t-test, $P < 0.05$).

Appendix C.
Expression Values and Statistical Data Tables

Tables containing relative expression values for genes, calculated by densitometric analysis, as well as descriptive statistics and statistical analysis results.

Abbreviations Used

DA: Densitometric Analysis value
df: Degrees of Freedom
F stat: ANOVA test statistic
RE: Relative Expression (the mean of all densitometric analyses as a percent)
SD: Standard Deviation
SEM: Standard Error of the Mean
t-stat: Student t-test statistic
P value: The smallest level of significance for which the observed sample statistic rejects the null hypothesis for the performed two-tailed t-test with a 5% level of significance ($\alpha = 0.05$).

Densitometric Analysis

The relative expression of each gene for a particular sample was calculated using the software QuantityOne® (BioRad) that calculated the volume of fluorescence of each band with background subtraction (% adjusted volume). The densitomentric analysis values were calculated by the following equation:

DA = (% adjusted volume of gene of interest) ÷ (% adjusted volume of the reference gene)

The mean DA value (average of all experimental trials) was multiplied by 100% to have the relative expressions as a whole number instead of a decimal.

Statistical Analyses

Student t-test (independent samples)

Assumptions: (1) Samples obtained from populations with normal distributions
(2) Samples obtained from populations with equal standard deviations

One-way ANOVA (Analysis of Variance)

Assumptions: (1) Samples obtained from populations with normal distributions
(2) Samples obtained from populations with equal standard deviations
(3) Samples are independent

Table 3. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in insect cuticle and bean root exudate (BRE) media. The fungal samples were incubated for 12 hours at room temperature in 1% media.

	Locust	**Cockroach**	**Tobacco Hornworm**	**Chitin**	**0.1% BRE**	**1% BRE**	**10% BRE**	**Tomato Stems**	**Cellulose**	**Starch**
Mad1										
DA #1	0.4504	0.3137	0.3777	0.1202	0.0807	0.1723	0.6657	0.0295	0.1530	0.3161
DA #2	0.3328	0.1333	0.3239	0.0686	0.0809	0.1364	0.2291	0.0839	0.1932	0.2254
DA #3	0.5477	0.3272	0.4462	0.0488	0.0836	0.2081	0.6748	0.0164	0.1597	0.3578
Mean	0.4437	0.2581	0.3826	0.0792	0.0817	0.1723	0.5232	0.0433	0.1686	0.2998
RE (%)	44.3650	25.8096	38.2598	7.9214	8.1726	17.2273	52.3219	4.3263	16.8643	29.9775
SD	10.7599	10.8285	6.1266	3.6843	0.1618	3.5863	25.4733	3.5800	2.1564	6.7698
SEM	6.2123	6.2519	3.5372	2.1271	0.0934	2.0706	14.7070	2.0669	1.2450	3.9085
Mad2										
DA #1	0.1503	0.3757	0.0830	0.4348	0.4295	0.6026	1.0064	0.1555	0.1285	0.3024
DA #2	0.3036	0.4149	0.2335	0.5342	0.7257	0.9459	1.1981	0.2187	0.4225	0.4283
DA #3	0.1627	0.4620	0.1097	0.2231	0.4389	0.5999	0.9348	0.1707	0.1489	0.3220
Mean	0.2055	0.4175	0.1420	0.3974	0.5314	0.7161	1.0464	0.1816	0.2333	0.3509
RE (%)	20.5505	41.7548	14.2028	39.7363	53.1411	71.6117	104.6411	18.1624	23.3333	35.0899
SD	8.5136	4.3180	8.0295	15.8898	16.8364	19.8974	13.6130	3.3000	16.4161	6.7758
SEM	4.9153	2.4930	4.6358	9.1740	9.7205	11.4878	7.8595	1.9053	9.4779	3.9120
t-stat	3.0063	2.3691	4.1256	3.3724	4.6259	4.6590	3.1375	4.9213	0.6767	0.9245
P value	*0.0397	0.0769	*0.0145	*0.0280	*0.0098	*0.0096	*0.0349	*0.0079	0.5357	0.4076

*P values indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression.

Table 4. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in healthy and damaged root exudates of asparagus, carrot and basil. The fungal samples were incubated for 12 hours at room temperature in 0.1 mg/mL exudate.

	Asparagus	Damaged Asparagus	Carrot	Damaged Carrot	Basil	Damaged Basil
Mad1						
DA #1	0.0076	0.0000	0.0000	0.0000	0.0000	0.0000
DA #2	0.0112	0.0000	0.0087	0.0000	0.0000	0.0000
DA #3	0.0011	0.0071	0.0131	0.0000	0.0107	0.0029
Mean	0.0066	0.0024	0.0073	0.0000	0.0036	0.0010
RE (%)	0.6621	0.2356	0.7267	0.0000	0.3553	0.0960
SD	0.5134	0.4081	0.6681	0.0000	0.6153	0.1663
SEM	0.2964	0.2356	0.3857	0.0000	0.3553	0.0960
Mad2						
DA #1	0.1097	0.0241	0.0863	0.0780	0.3401	0.7133
DA #2	0.1314	0.0333	0.1770	0.1357	0.2894	0.4859
DA #3	0.0671	0.0326	0.1298	0.0861	0.2003	0.3689
Mean	0.1027	0.0300	0.1310	0.0999	0.2766	0.5227
RE (%)	10.2726	3.0034	13.1039	9.9927	27.6625	52.2692
SD	3.2721	0.5119	4.5396	3.1279	7.0786	17.5108
SEM	1.8892	0.2956	2.6210	1.8059	4.0868	10.1098
t-stat	5.0257	7.3222	4.6721	5.5334	6.6567	5.1604
P value	*0.0074	*0.0019	*0.0095	*0.0052	*0.0027	*0.0067

*P values indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression.

Table 5. Statistical significance for difference in *Mad2* gene expression for *M. robertsii* grown in healthy and damaged root exudates of asparagus, carrot and basil. The fungal samples were incubated for 12 hours at room temperature in 0.1 mg/mL exudate.

ANOVA ($\alpha = 0.05$)			
	df	**F stat**	**P value**
Healthy exudates	2	7.9723	*0.0204
Damaged exudates	2	20.1928	*0.0022
t-test ($\alpha = 0.05$)			
	df	**t – stat**	**P value**
Healthy vs. Damaged			
Asparagus	4	1.3615	0.2450
Carrot	4	0.9778	0.3835
Basil	4	2.2567	0.0870

*P values indicating a significant difference ($\alpha = 0.05$) in *Mad2* gene expression.

Table 6. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) between the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in various carbohydrate media. The fungal samples were incubated for 12 hours at room temperature in 1% media.

	Glucose	**Trehalose**	**NAG**	**Raffinose**	**Maltose**	**Lactose**	**Arabinose**	**Sorbose**	**Fructose**
Mad1									
DA #1	0.1241	0.4914	0.0139	0.4212	0.1179	0.5301	0.6312	0.5918	0.5108
DA #2	0.0595	0.3420	0.0093	0.4048	0.0774	0.3797	0.5374	0.4439	0.4498
DA #3	0.1653	0.9783	0.0000	0.5262	0.1422	0.6252	0.7947	0.5724	0.7894
Mean	0.1163	0.6039	0.0077	0.4507	0.1125	0.5117	0.6544	0.5360	0.5833
RE	11.6310	60.3893	0.7720	45.0734	11.2486	51.1664	65.4444	53.6030	58.3350
SD	5.3352	33.2750	0.7061	6.5837	3.2747	12.3782	13.0177	8.0366	18.1004
SEM	3.0803	19.2113	0.4077	3.8011	1.8906	7.1465	7.5158	4.6399	10.4503
Mad2									
DA #1	0.0470	0.5680	0.0047	0.2345	0.5500	0.7239	0.4919	0.7612	0.5744
DA #2	0	0.4282	0.0026	0.4824	0.2033	0.6541	0.4283	0.6649	0.5488
DA #3	0.0440	0.7312	0.0000	0.5922	0.0657	0.6782	0.3909	0.7376	0.7543
Mean	0.0303	0.5758	0.0024	0.1678	0.5415	0.6854	0.4371	0.7212	0.6258
RE	3.0327	57.5804	0.2439	16.7825	54.1524	68.5385	43.7064	72.1223	62.5820
SD	2.6305	15.1663	0.2348	8.9807	5.5431	3.5480	5.1070	5.0176	11.1972
SEM	1.5187	8.7563	0.1356	5.1850	3.2003	2.0484	2.9485	2.8969	6.4647
t-stat	2.5036	0.1330	1.2294	1.8272	1.0027	2.3367	2.6925	3.3856	0.3456
P value	0.0665	0.9006	0.2863	0.1417	0.3727	0.0797	0.0545	*0.0276	0.7470

*P value indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression.

Table 7. Statistical significance (ANOVA, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in various carbohydrate media. The fungal samples were incubated for 12 hours at room temperature in 1% media.

ANOVA ($\alpha = 0.05$)			
	df	**F stat**	**P value**
Mad1	8	8.6883	*0.0001
Mad2	8	39.7178	*0.0000

*P value indicating a significant difference ($\alpha = 0.05$) in *Mad1* and *Mad2* gene expression between the various carbohydrate media.

Table 8. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) between the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in various carbohydrate media supplemented with peptone. The fungal samples were incubated for 12 hours at room temperature in 1% carbohydrate with 0.1% peptone media.

	Glucose-peptone	**Trehalose-peptone**	**NAG-peptone**	**Raffinose-peptone**	**Maltose-peptone**	**Lactose-peptone**	**Arabinose-peptone**	**Sorbose-peptone**	**Fructose-peptone**
Mad1									
DA #1	0.4617	0.3405	0.5726	0.6805	0.6992	0.1033	0.6036	0.2010	0.8121
DA #2	0.5032	0.4033	0.6013	0.6459	0.6332	0.1103	0.6022	0.2161	0.6906
DA #3	0.5479	0.4550	0.6099	0.7266	0.7546	0.1194	0.6453	0.2450	0.8552
Mean	0.5043	0.3996	0.5946	0.6843	0.6957	0.1110	0.6171	0.2207	0.7860
RE (%)	50.4282	39.9600	59.4622	68.4320	69.5674	11.0998	61.7069	22.0682	78.5997
SD	4.3127	5.7312	1.9525	4.0447	6.0784	0.8044	2.4488	2.2368	8.5378
SEM	2.4899	3.3089	1.1273	2.3352	3.5094	0.4644	1.4138	1.2914	4.9293
Mad2									
DA #1	0.0646	0.0403	0.4254	0.0449	0.0923	0.2281	0.5648	0.1515	0.1277
DA #2	0.0728	0.0449	0.4193	0.0578	0.0573	0.2350	0.5432	0.1559	0.0525
DA #3	0.0794	0.0489	0.3794	0.0667	0.1086	0.2353	0.5769	0.1753	0.1250
Mean	0.0723	0.0447	0.4080	0.0564	0.0861	0.2328	0.5617	0.1609	0.1017
RE (%)	7.2263	4.4698	40.8001	5.6441	8.6063	23.2811	56.1650	16.0862	10.1718
SD	0.7442	0.4283	2.4998	1.0982	2.6218	0.4112	1.7053	1.2659	4.2676
SEM	0.4297	0.2473	1.4432	0.6340	1.5137	0.2374	0.9845	0.7309	2.4639
t-stat	17.0981	10.6958	10.1907	25.9480	15.9505	23.3535	3.2167	4.0313	12.4171
P value	*0.0001	*0.0004	*0.0005	*0.0000	*0.0001	*0.0000	*0.0324	*0.0157	*0.0002

*P value indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression in various carbohydrate media supplemented with peptone.

Table 9. Statistical significance (ANOVA, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in various carbohydrate media supplemented with peptone. The fungal samples were incubated for 12 hours at room temperature in 1% carbohydrate with 0.1% peptone media.

ANOVA ($\alpha = 0.05$)			
	df	**F stat**	**P value**
Mad1	8	72.4844	*0.0000
Mad2	8	230.9255	**0.0000

*P value indicating a significant difference ($\alpha = 0.05$) in *Mad1* gene expression between the various carbohydrates supplemented with peptone.

**P value indicating a significant difference ($\alpha = 0.05$) in *Mad2* gene expression between the various carbohydrates supplemented with peptone.

Table 10. Statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in 1% carbohydrate media compared to 1% carbohydrate media supplemented with 0.1% peptone.

	Glucose-peptone	**Trehalose-peptone**	**NAG-peptone**	**Raffinose-peptone**	**Maltose-peptone**	**Lactose-peptone**	**Arabinose-peptone**	**Sorbose-peptone**	**Fructose-peptone**
Mad1									
t – stat	9.7953	1.0480	48.9614	5.2361	14.6300	5.5946	0.4887	6.5475	1.7538
P value	*0.0006	0.3538	*0.0000	*0.0064	*0.0001	*0.0050	0.6506	*0.0028	0.1543
Mad2									
t – stat	2.6570	6.0630	27.9775	14.8685	1.5137	21.9468	4.0078	18.7558	7.5756
P value	0.0566	**0.0037	**0.0000	**0.0001	0.2047	**0.0000	**0.0160	**0.0001	**0.0016

*P value indicating a significant difference ($\alpha = 0.05$) in *Mad1* gene expression between carbohydrate media and carbohydrate media supplemented with peptone.

**P value indicating a significant difference ($\alpha = 0.05$) in *Mad2* gene expression between carbohydrate media and carbohydrate media supplemented with peptone.

Table 11. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in sterile-distilled water over the course of 72 hours. The fungal samples were incubated at room temperature and removed at the indicated time intervals.

	Length of Growth in Sterile-Distilled Water (hours)							
	0.25	**2**	**4**	**8**	**12**	**24**	**48**	**72**
Mad1								
DA #1	0.1345	0.0257	0.1130	0.0460	0.0636	0.0088	0.0000	0.0156
DA #2	0.0546	0.0506	0.0003	0.2378	0.0001	0.0000	0.0000	0.0000
DA #3	0.0737	0.0554	0.0000	0.2382	0.0040	0.0000	0.0000	0.0000
Mean	0.0876	0.0439	0.0378	0.1740	0.0226	0.0029	0.0000	0.0052
RE (%)	8.7612	4.3905	3.7762	17.4025	2.2602	0.2928	0.0000	0.5199
SD	4.1755	1.5921	6.5174	11.0841	3.5599	0.5072	0.0000	0.9006
SEM	2.4107	0.9192	3.7628	6.3994	2.0553	0.2928	0.0000	0.5199
Mad2								
DA #1	0.0375	0.1424	0.6753	0.5208	0.4914	0.7481	0.5114	0.1313
DA #2	0.0480	0.3545	0.3583	0.7522	0.4369	0.6909	0.5139	0.1573
DA #3	0.0484	0.3436	0.3266	0.7404	0.4828	0.6549	0.5014	0.2111
Mean	0.0446	0.2802	0.4534	0.6711	0.4703	0.6980	0.5089	0.1666
RE (%)	4.4632	28.0172	45.3383	67.1132	47.0337	69.7955	50.8878	16.6594
SD	0.6147	11.9422	19.2795	13.0365	2.9269	4.6979	0.6599	4.0699
SEM	0.3549	6.8948	11.1310	7.5266	1.6898	2.7123	0.3810	2.3497
t-stat	1.7639	3.3967	3.5373	5.0318	16.8271	25.4767	133.5718	6.7064
P value	0.1528	*0.0274	*0.0241	*0.0073	*0.0001	*0.0000	*0.0000	*0.0026

*P value indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression during growth in sterile-distilled water at the respective time interval.

Table 12. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in minimal media M-100 over the course of 72 hours. The fungal samples were incubated at room temperature and removed at the indicated time intervals.

	Length of Growth Minimal Media M-100 (hours)							
	0.25	**2**	**4**	**8**	**12**	**24**	**48**	**72**
Mad1								
DA #1	0.0108	0.0585	0.0597	0.0621	0.1763	0.0758	0.0000	0.0209
DA #2	0.0551	0.2105	0.0323	0.5735	0.4199	0.0629	0.0000	0.0000
DA #3	0.0202	0.2082	0.0409	0.5167	0.3869	0.0746	0.0000	0.0000
Mean	0.0287	0.1591	0.0443	0.3841	0.3277	0.0711	0.0000	0.0070
RE (%)	2.8741	15.9070	4.4283	38.4069	32.7705	7.1110	0.0000	0.6978
SD	2.3347	8.7086	1.3981	28.0305	13.2155	0.7103	0.0000	1.2085
SEM	1.3479	5.0279	0.8072	16.1834	7.6299	0.4101	0.0000	0.6978
Mad2	0.0408	0.0844	0.3777	0.3550	0.7442	0.7057	0.3892	0.4227
DA #1	0.0626	0.1493	0.3373	0.6099	0.7931	0.7612	0.6259	0.7256
DA #2	0.0674	0.1525	0.3401	0.5939	0.7530	0.7412	0.6335	0.7084
DA #3	0.0570	0.1287	0.3517	0.5196	0.7635	0.7361	0.5495	0.6189
Mean	5.6951	12.8732	35.1733	51.9592	76.3461	73.6051	54.9514	61.8892
RE (%)	1.4170	3.8468	2.2575	14.2793	2.6083	2.8123	13.8884	17.0131
SD	0.8181	2.2209	1.3034	8.2441	1.5059	1.6237	8.0185	9.8225
SEM	0.8181	2.2209	1.3034	8.2441	1.5059	1.6237	8.0185	9.8225
t-stat	1.7891	0.5519	20.0546	0.7462	5.6030	39.7062	6.8531	6.2141
P value	0.1481	0.6104	*0.0000	0.4970	*0.0050	*0.0000	*0.0024	*0.0034

*P value indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression during growth in minimal media M-100 at the respective time interval.

Table 13. Densitometric analysis, descriptive statistics and statistical significance (t-test, $\alpha = 0.05$) for the relative gene expression of *Mad1* and *Mad2* for *M. robertsii* grown in YPD over the course of 72 hours. The fungal samples were incubated at room temperature and removed at the indicated time intervals.

	Length of Growth YPD (hours)							
	0[¥]	2	4	8	12	24	48	72
Mad1								
DA #1	0.5631	0.0639	0.0998	0.6675	0.2568	0.2986	0.1086	0.0000
DA #2	0.5652	0.0815	0.0687	0.6186	0.2290	0.2644	0.0000	0.0000
DA #3	0.5105	0.0705	0.0591	0.6267	0.1780	0.2559	0.0000	0.0000
Mean	0.5463	0.0760	0.0758	0.6376	0.2213	0.2730	0.0362	0.0000
RE (%)	37.8672	7.5966	7.5842	63.7592	22.1262	27.2951	3.6190	0.0000
SD	3.0989	0.8858	2.1271	2.6211	3.9936	2.2576	6.2684	0.0000
SEM	1.7891	0.5114	1.2281	1.5133	2.3057	1.3034	3.6190	0.0000
Mad2								
DA #1	0.4857	0.0338	0.1232	0.5762	0.3100	0.2270	0.0344	0.0000
DA #2	0.4636	0.0423	0.1119	0.6186	0.5532	0.1989	0.0000	0.0000
DA #3	0.4560	0.0374	0.1126	0.6260	0.3878	0.2072	0.0000	0.0000
Mean	0.4685	0.0398	0.1159	0.6069	0.4170	0.2110	0.0115	0.0000
RE (%)	34.3904	3.9850	11.5922	60.6927	41.6993	21.1010	1.1467	0.0000
SD	1.5427	0.4224	0.6352	2.6896	12.4216	1.4460	1.9861	0.0000
SEM	0.8907	0.2439	0.3667	1.5528	7.1716	0.8348	1.1467	0.0000
t-stat	3.8943	6.0215	3.1272	1.4143	2.5983	4.0017	0.6513	-
P value	*0.0176	*0.0038	*0.0353	0.2302	00612	*0.0612	*0.0161	-

[¥]Sample is from the four-day old inoculum culture not from a sample transferred to fresh YPD broth for 0.25 hours.

*P value indicating a significant difference ($\alpha = 0.05$) between *Mad1* and *Mad2* gene expression during growth in YPD at the respective time interval.

Table 14. Statistical significance (ANOVA $\alpha = 0.05$) for the change in *Mad2* relative gene expression over time for *M. robertsii* grown in water, minimal media M-100 and YPD over the course of 72 hours.

ANOVA ($\alpha = 0.05$)			
	df	**F stat**	**P value**
Water	8	17.6037	*0.0000
Minimal Media	8	23.1873	*0.0000
YPD	8	18.4580	*0.0000

*P value indicating a significant difference ($\alpha = 0.05$) in *Mad2* gene expression over time.

Table 15. Statistical significance (t-test, $\alpha = 0.05$) for the change in *Mad2* relative gene expression for *M. robertsii* grown in water, minimal media M-100 and YPD at the indicated time interval.

	Time Interval (hours)							
Water	**0 to 2**	**2 to 4**	**2 to 8**	**4 to 8**	**8 to 12**	**12 to 24**	**24 to 48**	**48 to 72**
t-stat	3.4117	1.3229	3.8302	1.6205	2.6030	7.1227	6.9032	14.3791
P value	*0.0270	0.2564	*0.0186	0.1804	0.0599	*0.0021	*0.0023	*0.0001
Minimal media	**0 to 2**	**2 to 4**	**4 to 8**	**4 to 12**	**8 to 12**	**12 to 24**	**24 to 48**	**48 to 72**
t-stat	3.0328	8.6598	2.0111	20.6729	2.9010	0.5876	2.2801	0.5472
P value	*0.0387	*0.0010	0.1147	*0.0000	*0.0437	0.5981	0.0848	0.6134
YPD	**0 to 2**	**2 to 4**	**2 to 4**	**4 to 8**	**8 to 12**	**12 to 24**	**24 to 48**	**48 to 72**
t-stat	46.6308	17.7291	17.7291	30.7738	2.5884	22.4570	14.0685	1.0000
P value	*0.0000	*0.0001	*0.0001	*0.0000	0.0608	*0.0000	*0.0002	0.3739

*P value indicating a significant difference ($\alpha = 0.05$) in *Mad2* gene expression for the indicated time interval of the respective media.

Table 16. Densitometric analysis and descriptive statistics for the relative gene expression of *Mad2* for *M. robertsii* grown in YPD-based and water-based stress media for 2 hours at room temperature (except when noted otherwise).

	Mad2											
YPD - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.1056	0.0334	0.0204	0.1083	0.0000	0.0487	0.0116	0.1039	0.0016	0.0407	0.0168	0.3617
DA #2	0.0978	0.0225	0.0298	0.0643	0.0000	0.0337	0.0478	0.0391	0.0000	0.0262	0.0044	0.3870
DA #3	0.0878	0.0500	0.0243	0.0526	0.0000	0.0240	0.0618	0.0483	0.0232	0.0000	0.0121	0.3746
MEAN	0.0971	0.0353	0.0248	0.0751	0.0000	0.0354	0.0404	0.0638	0.0083	0.0223	0.0111	0.3744
RE (%)	9.7061	3.5284	2.4815	7.5064	0.0000	3.5439	4.0403	6.3779	0.8277	2.2286	1.1114	37.4396
SD	0.8923	1.3841	0.4699	2.9349	0.0000	1.2440	2.5882	3.5027	1.2972	2.0621	0.6243	1.2654
SEM	0.5152	0.7991	0.2713	1.6944	0.0000	0.7182	1.4943	2.0223	0.7490	1.1906	0.3604	0.7306
Water - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.9566	1.1097	0.6420	1.1505	1.1640	1.1896	1.3952	1.0292	0.9131	0.5418	0.9354	0.8379
DA #2	0.9950	1.2119	0.7126	1.4564	0.8591	1.1000	1.1659	1.0425	0.7538	0.6510	0.9612	1.0483
DA #3	1.1314	1.3100	1.1744	0.7434	1.2217	1.0149	1.1044	1.2257	1.0986	0.8041	0.9928	0.9478
MEAN	1.0277	1.2105	0.8430	1.1168	1.0816	1.1015	1.2218	1.0991	0.9218	0.6656	0.9631	0.9447
RE (%)	102.766	121.054	84.302	111.677	108.162	110.150	122.183	109.913	92.185	66.561	96.312	94.468
SD	9.1876	10.0156	28.9138	35.7709	19.4866	8.7366	15.3269	10.9800	17.2597	13.1758	2.8783	10.5256
SEM	5.3045	5.7825	16.6934	20.6524	11.2506	5.0441	8.8490	6.3393	9.9649	7.6071	1.6618	6.0770

Table 17. Densitometric analysis and descriptive statistics for the relative gene expression of *ssgA* for *M. robertsii* grown in YPD-based and water-based stress media for 2 hours at room temperature (except when noted otherwise).

	ssgA											
YPD - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DA #2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DA #3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MEAN	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
RE (%)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SD	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SEM	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Water - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.2376	0.5593	0.2686	0.2367	0.4791	0.4243	0.2237	0.2950	0.2918	0.2455	0.2464	0.3333
DA #2	0.2713	0.5302	0.2660	0.2273	0.4795	0.3916	0.2601	0.3138	0.3137	0.2932	0.2809	0.2726
DA #3	0.2444	0.5399	0.2679	0.2647	0.4874	0.4037	0.2436	0.2569	0.2806	0.2693	0.2484	0.3630
MEAN	0.2511	0.5431	0.2675	0.2429	0.4820	0.4065	0.2425	0.2886	0.2954	0.2693	0.2586	0.3230
RE (%)	25.1106	54.3146	26.7482	24.2901	48.2004	40.6533	24.2466	28.8566	29.5371	26.9337	25.8586	32.2959
SD	1.7809	1.4827	0.1337	1.9458	0.4705	1.6534	1.8223	2.8976	1.6853	2.3815	1.9353	4.6068
SEM	1.0282	0.8560	0.0772	1.1234	0.2717	0.9546	1.0521	1.6729	0.9730	1.3749	1.1173	2.6597

Table 18. Densitometric analysis and descriptive statistics for the relative gene expression of *Hsp*30 for *M. robertsii* grown in YPD-based and water-based stress media for 2 hours at room temperature (except when noted otherwise).

	*Hsp*30											
YPD - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.0000	0.3710	0.2905	0.1972	0.0906	0.0617	0.1156	0.1027	0.0000	0.1138	0.1073	0.1107
DA #2	0.0000	0.4053	0.3189	0.2531	0.1369	0.1249	0.0782	0.0268	0.0372	0.0716	0.0742	0.1377
DA #3	0.0000	0.3864	0.3657	0.2583	0.0924	0.0000	0.0506	0.0000	0.0878	0.0101	0.0582	0.1161
MEAN	0.0000	0.3876	0.3250	0.2362	0.1066	0.0622	0.0815	0.0432	0.0417	0.0652	0.0799	0.1215
RE (%)	0.0000	38.7553	32.5050	23.6215	10.6643	6.2181	8.1460	4.3152	4.1674	6.5172	7.9906	12.1477
SD	0.0000	1.7177	3.8002	3.3910	2.6201	6.2431	3.2591	5.3247	4.4060	5.2163	2.5031	1.4266
SEM	0.0000	0.9917	2.1941	1.9578	1.5127	3.6045	1.8817	3.0742	2.5438	3.0116	1.4452	0.8237
Water - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.8454	0.7606	0.9095	0.8454	0.7721	0.8228	0.6628	0.7718	0.7544	0.7021	0.6523	0.7443
DA #2	0.8136	0.8674	0.9516	0.8600	0.8008	0.8546	0.6279	0.7289	0.7634	0.6869	0.6447	0.6301
DA #3	0.8278	0.8481	0.9399	0.8443	0.8484	0.8322	0.6941	0.6787	0.7007	0.7241	0.7795	0.7397
MEAN	0.8289	0.8254	0.9337	0.8499	0.8071	0.8365	0.6616	0.7265	0.7395	0.7044	0.6922	0.7047
RE (%)	82.8915	82.5355	93.3652	84.9913	80.7121	83.6531	66.1570	72.6466	73.9475	70.4392	69.2193	70.4702
SD	1.5925	5.6937	2.1732	0.8786	3.8548	1.6331	3.3109	4.6596	3.3895	1.8717	7.5703	6.4661
SEM	0.9194	3.2873	1.2547	0.5073	2.2256	0.9429	1.9116	2.6902	1.9569	1.0806	4.3707	3.7332

Table 19. Densitometric analysis and descriptive statistics for the relative gene expression of *Hsp70* for *M. robertsii* grown in YPD-based and water-based stress media for 2 hours at room temperature (except when noted otherwise).

	Hsp70											
YPD - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.0328	0.1862	0.4865	0.1261	0.0603	0.0257	0.0870	0.0000	0.0629	0.0000	0.1284	0.1364
DA #2	0.0000	0.1570	0.4957	0.1806	0.0482	0.0000	0.0424	0.0000	0.0115	0.0000	0.1597	0.1375
DA #3	0.0410	0.1618	0.4968	0.1752	0.0549	0.0000	0.0102	0.0000	0.1278	0.0000	0.1478	0.1430
MEAN	0.0246	0.1684	0.4930	0.1607	0.0545	0.0086	0.0465	0.0000	0.0674	0.0000	0.1453	0.1390
RE (%)	2.4620	16.8363	49.2997	16.0663	5.4468	0.8582	4.6529	0.0000	6.7396	0.0000	14.5315	13.8959
SD	2.1710	1.5669	0.5614	3.0032	0.6067	1.4865	3.8541	0.0000	5.8275	0.0000	1.5790	0.3537
SEM	1.2534	0.9047	0.3241	1.7339	0.3503	0.8582	2.2252	0.0000	3.3645	0.0000	0.9117	0.2042
Water - base	**15°C**	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**1.5M KCl**	**0.5M sorbitol**	**2M sorbitol**	**10mM H_2O_2**	**100mM H_2O_2**	***Manduca* cuticle**
DA #1	0.2545	0.4399	0.7528	0.5142	0.2802	0.3974	0.3252	0.3337	0.3885	0.3839	0.4160	0.4057
DA #2	0.2614	0.4861	0.7114	0.5642	0.3067	0.3791	0.3294	0.3293	0.3924	0.3708	0.4213	0.3951
DA #3	0.2575	0.4343	0.7615	0.5158	0.2957	0.3767	0.3435	0.3723	0.4063	0.3585	0.3852	0.4065
MEAN	0.2578	0.4534	0.7419	0.5314	0.2942	0.3844	0.3327	0.3451	0.3957	0.3711	0.4075	0.4024
RE (%)	25.7815	45.3433	74.1931	53.1428	29.4198	38.4400	33.2728	34.5084	39.5715	37.1068	40.7508	40.2422
SD	0.3410	2.8432	2.6749	2.8402	1.3277	1.1350	0.9603	2.3679	0.9345	1.2692	1.9467	0.6362
SEM	0.1969	1.6415	1.5443	1.6398	0.7666	0.6553	0.5544	1.3671	0.5395	0.7328	1.1239	0.3673

Table 20. Statistical analysis (t-test, $\alpha = 0.05$) of *Mad2*, *ssgA*, *Hsp*30, and *Hsp*70 gene expression for *M. robertsii* exposed to different concentrations of KCl, sorbitol and hydrogen peroxide for 2 hours in YPD or water.

		0.7M and 1.5M KCl		0.5M and 2M Sorbitol		10mM and 100mM H_2O_2	
		t – stat	P value	t – stat	P value	t – stat	P value
Mad2	YPD	0.2994	0.7795	2.5737	0.0617	0.8981	0.4199
	water	1.1272	0.3227	2.0439	0.1105	0.2927	0.7843
ssgA	YPD	-	-	-	-	-	-
	water	2.3327	0.0800	1.5456	0.1971	2.2314	0.0895
***Hsp*30**	YPD	1.0628	0.3478	0.5961	0.5832	2.4991	0.0668
	water	1.9664	0.1207	1.5694	0.1916	0.2176	0.8384
***Hsp*70**	YPD	2.0910	0.1047	2.0031	0.1157	0.6803	0.5337
	water	0.8376	0.4494	2.7085	0.0536	0.4302	0.6892

Note: No significant differences thus only the lower concentration of each stress will be included in the remaining statistical analyses.

Table 21. Statistical analysis (ANOVA, $\alpha = 0.05$) of the relative gene expression of *Mad2, ssgA, Hsp*30, and *Hsp*70 for *M. robertsii* grown in YPD-based or water-based stress media for 2 hours.

		ANOVA ($\alpha = 0.05$)			
Gene	**Media Base**	**df**	**F stat**	**P value**	**Result**
Mad2	YPD	8	112.4428	0.0000	Expression varies between YPD-based media
	Water	8	2.0856	0.0931	Expression does not vary between water-based media
ssgA	YPD	-	-	-	Expression does not vary between YPD-based media
	Water	8	227.8327	0.0000	Expression varies between water-based media
*Hsp*30	YPD	8	39.2423	0.0000	Expression varies between YPD-based media
	Water	8	26.2320	0.0000	Expression varies between water-based media
*Hsp*70	YPD	8	66.0075	0.0000	Expression varies between YPD-based media
	Water	8	217.7913	0.0000	Expression varies between water-based media

Table 22. Statistical analysis (t-test, $\alpha = 0.05$) for the difference in *Mad2, ssgA, Hsp*30 and *Hsp*70 relative gene expression for *M. robertsii* grown for 2 hours under each stress condition in YPD-based media compared to water-based media.

	Stress Condition: Comparison of YPD- and Water-based Media								
	15°C	**22°C**	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**0.5M sorbitol**	**10mM H_2O_2**	***Manduca* cuticle**
Mad2									
t – stat	17.4615	20.1330	4.9007	8.8469	21.8375	13.3631	8.2438	46.0232	14.2484
P value	*0.0001	*0.0000	*0.0080	*0.0009	*0.0000	*0.0002	*0.0012	*0.0000	*0.0001
ssgA									
t – stat	24.4222	63.4484	346.4958	177.4236	42.5867	23.0461	32.4526	23.1434	20.2542
P value	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000
***Hsp*30**									
t – stat	90.1553	12.7506	24.0794	26.0303	20.7838	21.6274	21.7422	51.8872	10.0399
P value	*0.0000	*0.0002	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0006
***Hsp*70**									
t – stat	18.3795	15.2094	15.7753	28.4450	34.8047	12.4804	13.1289	29.1101	4.3917
P value	*0.0001	*0.0001	*0.0001	*0.0000	*0.0000	*0.0002	*0.0002	*0.0000	*0.0118

*P value indicating a significant difference ($\alpha = 0.05$) in gene expression between YPD-based and water-based media.

Table 23. Statistical analysis (t-test, $\alpha = 0.05$) of the relative gene expression of *Mad2, ssgA, Hsp*30 and *Hsp*70 for *M. robertsii* grown for 2 hours under each stress condition compared to the room temperature condition.

		Stress Conditions Compared to Room Temperature Condition							
		YPD-Based Stress Media							
		15°C	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**0.5M sorbitol**	**10mM H_2O_2**	***Manduca* cuticle**
Mad2	t-stat	6.4975	1.2406	2.1234	4.4155	0.0144	2.4788	0.9065	31.3196
	P value	*0.0029	0.2826	0.1010	*0.0116	0.9892	0.0683	0.4160	*0.0000
***Hsp*30**	t-stat	39.0796	2.5959	15.5300	8.7035	14.3904	12.6683	28.0983	6.1366
	P value	*0.0000	0.0603	*0.0001	*0.0010	*0.0001	*0.0002	*0.0000	*0.0036
***Hsp*70**	t-stat	9.2991	33.7817	11.7406	12.8136	5.0722	3.4747	1.7950	0.3408
	P value	*0.0001	*0.0000	*0.0003	*0.0002	*0.0071	*0.0255	0.1472	0.7504
		Water-Based Stress Media							
		15°C	**37°C**	**pH 4**	**pH 10**	**0.7M KCl**	**0.5M sorbitol**	**10mM H_2O_2**	***Manduca* cuticle**
ssgA	t-stat	21.8282	32.0720	6.8078	10.6544	22.1682	19.1187	20.2166	36.2731
	P value	*0.0000	*0.0000	*0.0024	*0.0004	*0.0000	*0.0000	*0.0000	*0.0000
***Hsp*30**	t-stat	0.1043	3.0779	0.4593	0.3268	4.3071	2.2449	5.4371	7.8892
	P value	0.9220	*0.0370	0.6699	0.7602	*0.0126	0.0882	*0.0056	*0.0014
***Hsp*70**	t-stat	11.8321	12.8001	8.7893	3.9057	6.9666	3.3403	1.9730	7.5640
	P value	*0.0003	*0.0002	*0.0009	*0.0175	*0.0022	*0.0288	0.1198	*0.0016

*P value indicating a significant difference ($\alpha = 0.05$) in gene expression between stress condition and room temperature condition.

Appendix D.
Mad2-GFP Transformant Procedures.

A detailed description of the *Mad2*-GFP construct procedure.

1. Double-Joint PCR (Procedure adopted from Yu et al. (2004))

i. Amplification of Fragments

The promoter and terminator sequences of *Mad2* were individually amplified, as was the open reading frame (ORF) of an engineered form of the green fluorescent protein (EGFP) from the jellyfish *Aequorea victoria*. The plasmid containing eGFP that has enhanced fluorescence was previously created in our lab and named pSK-GFP (Dryburgh 2008). Primers were designed using Primer Select DNAStar Lasergene software and are shown in Table I. The 3' end of the lower promoter primer has 3 nucleotides that match the first 3 nucleotides of the 5' end of the upper GFP ORF to enable fusion. The 5' end of the upper terminator primer has a guanine nucleotide to match the guanine nucleotide at the 3' end of the GFP lower primer to enable fusion. Two distinct restriction enzyme sites were included (one in the upper promoter primer and one in the lower terminator primer) for later ligation into a plasmid vector. Three separate 50 uL PCR reactions were carried out to amplify each sequence individually.

Table I: Double-Joint PCR Oligonucleotides

Primer	Sequence (5'→3')	Size (bp)
Mad2-Pro-U	TA***G GAT CC***[1] T CTG TCA GCT AGG AGA AGC	979 bp
Mad2-Pro-L	CCT CGC CCT TGC TCA CCA TTT TTA GAC GGA GTG TAG TAA ATG	
EGFP – U	ATG GTG AGC AAG GGC GAG GAG	1000 bp
EGFP – L	TTA CTT GTA CAG CTC GTC CAT GCC G	
Mad2-Term-U	GGA CGA GCT GTA CAA GTA AGC CGG GAG GGC GTG TTG ACC	717 bp
Mad2-Term-L	AT***C TCG AG***[2] A CAC CTC CGC GTC CAC AGA CAT ACC	

Note: [1]***BamH1 restriction site***, [2]***Xho1 restriction site***

Table II: Double-Joint PCR Promoter and Terminator Amplification Recipe

Recipe	Promoter	Terminator
Molecular Water	37 ul	37 ul
10X JumpStart Buffer	5 ul	5 ul
dNTP mix (10mM)	3 ul	3 ul
M. robertsii genomic DNA (100ng)	2 ul	2 ul
Forward (U) Primer	1 ul	1 ul
Reverse (L) Primer	1 ul	1 ul
JumpStart AccuTaq LA	1 ul	1 ul
Total volume	***50 ul***	***50 ul***

Table III: Double-Joint PCR EGFP Open Reading Frame Amplification Recipe

Recipe	GFP
Molecular Water	38.5 ul
10X JumpStart Buffer	5 ul
dNTP mix (10mM)	3 ul
Purified Plasmid pSK-EGFP	0.5 ul
EGFP-U Primer	1 ul
EGFP-L Primer	1 ul
JumpStart AccuTaq LA	1 ul
Total volume	***50 ul***

The PCR conditions were: 96°C for 1 minute, 30 cycles of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension 72°C for 5 minutes.

ii. Verification and Purification of Fragments

A 5 uL sample of each amplicon was resolved by electrophoresis on a 1% agarose gel for 35 minutes at 100 volts (V) to verify the amplification of the samples with the expected sizes: promoter amplicon 979 bp, terminator amplicon 1,000 bp and EGFP amplicon 717 bp. Verified fragments were extracted from the gel using the GenElute™ Gel Extraction Kit (Sigma) as per the manufacturer's instructions.

iii. Fusion and Amplification of Fragments

A concentration ratio of 1:3:1 for promoter:EGFP:terminator amplicons was prepared by calculating the concentration of each amplicon spectrophotometrically (NanoVue, GE) and making appropriate dilutions using molecular grade water. The fusion of the fragments was accomplished by PCR with the recipe found in Table IV and with the following conditions: 96°C for 1 minute, 15 cycles of 95°C for 15 seconds, 60°C for 45 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes.

Table IV: Double-Joint PCR Fragment Fusion Recipe

Reagent	Volume
Water	14.25 ul
10X JumpStart Buffer	2.0 ul
dNTP mix (10mM)	2.0 ul
Promotor fragment	1.0 ul
Terminator fragment	1.0 ul
EGFP fragment	1.0 ul
JumpStart AccuTaq LA	0.25 ul
Total Volume	***25 ul***

The fused product was amplified by PCR with the recipe found in Table V and with the following conditions: 96°C for 1 minute, 30 cycles of 95°C for 15 seconds, 65°C for 45 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes.

Table V: Double-Joint PCR Fused Product Amplification Recipe

Reagent	Volume
Water	37.5 ul
10X Buffer	5.0 ul
dNTP mix (10mM)	4.0 ul
First Reaction	1.0 ul
Oligo Mad2-Pro-U	1.0 ul
Oligo Mad2-Term-L	1.0 ul
JumpStart AccuTaq LA	0.5 ul
Total Volume	***50 ul***

iv. Verification of Construct Amplification

A 5 uL sample of the amplified fusion product (Mad2-GFP construct) was resolved by electrophoresis on a 1% agarose gel for 35 minutes at 100V to verify the Mad2-GFP amplicon size: 2,696 bp.

v. Verification of Gene Replacement

Using two sets of primers: Mad2-Pro-U + EGFP-L and EGFP-U + Mad2-Term-L, two PCR reactions were performed to confirm that the promoter and GFP sequences amplified together (1,696 bp) and that the GFP and terminator sequences amplified together (1,717 bp). This verified the sequences were in the correct order and had fused correctly. The reaction mix for each PCR is shown in Table VI and the PCR conditions were as follows: 96°C for 1 minute, 15 cycles of 95°C for 15 seconds, 65°C for 45 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes. A 5 uL aliquot of each reaction product was resolved by electrophoresis on a 1% agarose gel to verify the amplicon sizes.

Table VI: Double-Joint PCR Gene Replacement Verification Recipe.

Reagent	Volume
Water	17.0 ul
10X JumpStart Buffer	2.5 ul
dNTP mix (10mM)	1.0 ul
Upper Primer	1.0 ul
Lower Primer	1.0 ul
Mad2-GFP Construct [C]	2.0 ul
JumpStart AccuTaq LA	0.5 ul
Total Volume	***25 ul***

2. Mad2-GFP Construct Ligation into Plasmid

i. Purification of Mad2-GFP Construct and Plasmid

The Mad2-GFP PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's instructions. The plasmid pBARGEM7-2 (Pall and Brunelli 1993, obtained from the Fungal Genetics Stock Center, Missouri) was purified from *E. coli* cells (strain DH5α) using the GenElute Plasmid MiniPrep Kit (Sigma) as per the manufacturer's instructions.

ii. Digestion

The concentration of the purified Mad2-GFP construct and the purified plasmid was spectrophotometrically calculated in order to approximate equal concentrations for the digestion. The plasmid digestion reaction is shown in Table VII. Each reaction was incubated at 37°C for 5 hours.

Table VII: Mad2-GFP Construct and Plasmid Digestion Recipe.

	Mad2-GFP	Plasmid
Molecular Water	31.5 ul	18.5 ul
Digest	7.0 ul	20.0 ul
BSA (100X)	0.5 ul	0.5 ul
NE Buffer 3	5.0 ul	5.0 ul
BamH1	3.0 ul	3.0 ul
Xho1	3.0 ul	3.0 ul
Total Volume	***50 ul***	***50ul***

iii. Verification and Purification

25 uL of the digested plasmid and the digested Mad2-GFP construct were run on a 1% agarose gel for 40 minutes at 80V. The digests were purified using the GenElute Gel Extraction Kit (Sigma) as per the manufacturer's instructions. A second electrophoresis using 5 uL of each sample was run to verify the products were not lost after the purification.

iv. Ligation of Mad2-GFP into pBARGEM7-2 Plasmid

Four ligation conditions were performed at 16°C overnight (approximately 16 hours) to ensure the production of transformants (See Table VIII). Condition #1 was a control to verify the vector did not self ligate. A map of the pBARGEM7-2 plasmid is shown in Figure 14.

Table VIII: Mad2-GFP Ligation Reaction Conditions

	Condition 1	**Condition 2**	**Condition 3**	**Condition 4**
T4 Buffer	2 ul	2 ul	2 ul	2 ul
Vector	5 ul	5 ul	5 ul	5 ul
Fragment	-	2.5 ul	5 ul	10 ul
ATP	1 ul	1 ul	1 ul	1 ul
T4 DNA Ligase	1 ul	1 ul	1 ul	1 ul
H_2O	11 ul	8.5 ul	6 ul	1 ul
Total Volume	***20 ul***	***20 ul***	***20 ul***	***20 ul***

3. Bacterial Transformation

i. Transformation of E. coli (Method adopted from Froger and Hall 2007)

In a 2 mL micro-centrifuge tube, 25ul of competent JM109 *E. coli* cells (NEB) and 2.5 uL of the ligation were combined. One tube was prepared for each condition. The tube was mixed by tapping and incubated on ice for 20 minutes. The cells were heat-shocked by incubation at 42°C for 1 minute and then immediately place on ice for 2 minutes. 600ul of SOC medium (for 1L: 20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 0.19 g KCl, 4 mL 1M NaOH, 5 mL 2M MgCl2, 3.6 g glucose) was added and the tube was incubated at 37°C for 90 minutes. The tube was then incubated at 4°C for 2 minutes and the entire contents were spread over Luria-Bertani (LB) agar that contained Ampicillin (amp; 1 μL/mL) to select for transformants. The plates were sealed and incubated at 37°C for 24 hours.

ii. Plasmid Purification and Verification

A colony that originated from ligation condition #4 was transferred to 5 mL of LBamp broth and left to grow overnight at 25°C. The plasmid was harvested from these *E. coli* cells using the GenElute Plasmid Mini Prep Kit (Sigma) as per the manufacturer's instructions. An aliquot of the purified plasmid was digested with BamH1 and Xho1, as previously described, and was analyzed by 1% agarose gel electrophoresis for the presence of two bands corresponding to the plasmid and the construct.

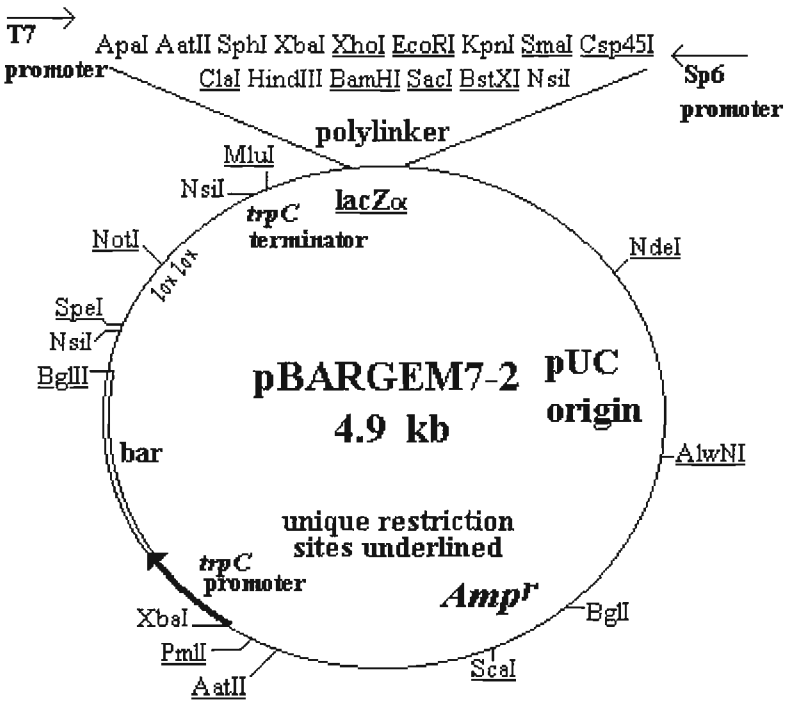

Figure 14: Map of the plasmid pBARGEM7-2 obtained from the Fungal Genetics Stock Center (FGSC) (Pall and Brunelli 1993).

4. Metarhizium Protoplast Transformation

Methods for transforming *Metarhizium* using protoplasts were adopted from procedures by Goettel et al. (1990) and Wang and St. Leger (2006).

50 mL of YPD was inoculated with 200 uL of a *Metarhizium* 2575 conidial suspension ($1x10^7$conidia/mL) and incubate for 12 hours at 27°C on a rotary shaker (~30 rpm). Germlings were collected by centrifugation (5,000 g for 5 minutes) and washed twice with sterile water and digested in 5 mL of Lysing Buffer + Lysing enzmes (10mM Tris pH 7.5; 1.2M sorbitol + dissolved in 10 mg/mL lysing enzymes from *Trichoderma harzianum* (Sigma)) at 27°C for 3 hours with gentile agitation.

Protoplasts were harvested by passing through nylon wool in a 5 mL syringe and collected by centrifugation at 1,500 g for 10 minutes. The pellet was washed twice with Transformation Buffer (10mM Tris pH 7.5, 1.2M Sorbitol, 20mM $CaCl_2$) and re-suspended in 100 uL Transformation Buffer to a concentration of ~2 x 10^7 cells/mL. The protoplasts (100 uL) were mixed with 15-20 ug of plasmid and left on ice for 30 minutes. After 30 minutes, 25ul of 60% polyethylene glycol (PEG) was added and the tube was kept on ice for 10 minutes. Another 500uL of 60% PEG was added and the tube was incubated at room temperature for 10 minutes. The suspension was diluted with 5 mL Regeneration Buffer (Glucose 10 g/L, $NaNO_3$ 6 g/L, KCl 0.52 g/L, $MgSO_4$:$7H_2O$ 0.52 g/L, KH_2PO_4 0.25 g/L, 1.2M sorbitol) and incubated for 18 hours at 27°C.

The suspension was mixed with 50 mL of 1.2% agar in Regeneration Buffer containing 160 ug/mL glufosinate ammonium, and five 10 mL plates were poured and incubated at 27°C. After 2-5 days the emerged colonies were transferred to M-100 media containing 160 ug/mL glufosinate ammonium. The putative transformants were verified by PCR using the primers Mad2-Pro-U with EGFP-L and EGFP-U with Mad2-Term-L.

Fluorescence microscopy was performed using a Leitz Diaplan microscope equipped with light filters suitable for blue light excitation of eGFP (Leitz I2/3; excitation filter: 450–490nm, dichroic mirror: 510nm, emission filter: 515nm). eGFP has an excitation maximum of 488nm and an emission maximum of 507nm (Cormack, Valdivia & Falkow, 1996).

Stability of the transformant was verified by subculturing five times on PDA plates. A culture was maintained on selective media (glufosinate ammonium infused M-100 agar).